ABSTRACT OF THESIS

Name of Candidate GEORGE KENNETH SCOTT

Address Department of Molecular Biology, University of Edinburgh.

Degree Doctor of Philosophy *Date* 20th September, 1972.

Title of Thesis THE EVOLUTION OF BACTERIAL PENICILLINASES

An improved procedure permitted the large scale, 430-fold purification of the β-lactamase (penicillinase) from Escherichia coli W3310, which carried the TEM resistance transfer factor. Physical, chemical and enzymological properties of the enzyme were determined.

Studies on the purified protein have yielded six fragments, accounting for about 100 residues (60%) of the amino-acid sequence. A 29-residue fragment was shown to be similar to regions of the Staphylococcus aureus and Bacillus licheniformis β-lactamase sequences, being about 35% identical. None of the other fragments exhibited any such detectable similarities.

The enzyme was shown to be partially inactivated by the specific nitration of a single tyrosine residue with tetranitromethane. The chymotryptic peptide which contained this residue was partially characterised. The enzyme was shown to be completely inactivated by the specific carboxymethylation of a single histidine residue. The corresponding tryptic peptide was sequenced and was shown to be very similar to a tryptic peptide from B.licheniformis β-lactamase.

The study of the variation of enzymic activity with pH permitted a tentative assignment of roles to the tyrosine and histidine residues in a mechanism of action of the hydrolysis of penicillin by β-lactamase.

These results are discussed with respect to the properties of β-lactamases from other species of bacteria.

Use other side if necessary.

THE EVOLUTION OF BACTERIAL PENICILLINASES

by

George Kenneth Scott

A thesis presented for the degree of Doctor of Philosophy of the University of Edinburgh.

Department of Molecular Biology,
University of Edinburgh.

September, 1972.

PREFACE

This thesis describes experimental work carried out by the author during the past three years. Appendix II summarises experiments with nucleases from Bacillus licheniformis, performed during a few months in 1969/70. The main body of the thesis relates to experiments performed between June, 1970 and July, 1972 with a β-lactamase from Escherichia coli.

I am very grateful to Dr. R.P. Ambler, who supervised this project, for his instruction, advice, encouragement and patience. I am also grateful to Professor M.R. Pollock for his continuing interest in this work. I would like to thank numerous members of this department for advice and assistance, and especially Dr. J.W. Melling of the Microbiological Research Establishment, who carried out the preliminary purification of the β-lactamase, Mrs. A.P. Ambler, of the Department of Machine Intelligence and Perception, who ran the computer comparison (Chapter IX) and Miss S.M. Murray, who attempted the automatic sequential degradation of the β-lactamase. Finally, I would like to thank my wife, Charlotte, for her patience and understanding.

Except as noted above, the experimental work described is my own. Appendix I is a pre-print of a joint paper by Dr. Melling and myself, describing the full purification of the β-lactamase.

SUMMARY

An improved procedure permitted the large scale 430-fold purification of the β-lactamase (penicillinase) from Escherichia coli W3310, which carried the TEM resistance transfer factor. Physical, chemical and enzymological properties of the enzyme were determined.

Studies on the purified protein have yielded six fragments, accounting for about 110 residues (60%) of the amino-acid sequence. A 29-residue fragment was shown to be similar to regions of the Staphylococcus aureus and Bacillus licheniformis β-lactamase sequences, being about 35% identical. None of the other fragments exhibited any such detectable similarities.

The enzyme was shown to be partially inactivated by the specific nitration of a single tyrosine residue with tetranitromethane. The chymotryptic peptide which contained this residue was partially characterised. The enzyme was shown to be completely inactivated by the specific carboxymethylation of a single histidine residue. The corresponding tryptic peptide was sequenced and was shown to be very similar to a tryptic peptide from B.licheniformis β-lactamase.

The study of the variation of enzymic activity with pH permitted a tentative assignment of roles to the tyrosine and histidine residues in a mechanism of action of the hydrolysis of penicillin by β-lactamase.

These results are discussed with respect to the properties of β-lactamases from other species of bacteria.

CONTENTS

CHAPTER I.

INTRODUCTION

Most biologists nowadays accept the Neo-Darwinian view of evolution (Hamilton, 1967). Mutation and re-organisation in the genetic material introduces and maintains a degree of variability in all organisms. Environmental conditions favour the propagation of suitably-adapted variants, this process being known as natural selection. Reproductive isolation of differently-adapted populations gives rise to speciation.

There is evidence, both from the observation of plants and animals in the wild and from laboratory experiments (Hamilton, 1967), that natural selection can act upon single, defined characteristics. Also, hybridisation between organisms from differently-adapted populations of the same species gives complex segregation of characteristics in the second generation which indicates that a large number of relatively small genetic differences distinguish the original populations. It would seem reasonable, from this evidence, to conclude that selection strong enough to favour the slightest advantageous mutation is the driving force in evolution, and that the concomitant mutational steps are relatively small. Major mutational changes such as gene duplication, which produces new genetic material for future development, must sometimes occur. However, the possibility that many mutations may be insignificant and are propagated by chance association with "selected" mutations must also be considered.

The comparison of the amino-acid sequences of proteins has a dual significance in evolutionary studies. These sequences reflect

corresponding base sequences in DNA and so give insight into the extent of mutation. They are also responsible for the structure and function of proteins, and so the effects of mutation can, in theory, be determined. This comparative aspect dates back to very early protein sequence determination (Harris et al., 1956).

The present-day techniques in protein sequence determination have been reviewed many times. Briefly, the purified protein is independently fragmented by different, specific, proteolytic enzymes or by specific chemical methods. Small, purified fragments are analysed and sequentially degraded so as to yield a (partial) sequence. Overlapping fragments are fitted into the complete sequence. Using this method, the sequence determination for a protein of molecular weight up to 50,000 daltons should be relatively straightforward, although several grams of highly-purified material may be necessary; subsequently, homologous sequences can be tackled more easily. Theoretically, bigger proteins should involve a linear increase in effort but the imperfect specificity of proteases leads to much more complex mixtures of peptides. Thus peptide purification becomes more difficult and the overall yields of pure peptides are smaller.

An automated, sequential degradation procedure for proteins, based on the "Edman-PITC" technique, is now commercially available (Edman, 1970). Starting from the N-terminus, the sequence of the first forty or more amino-acid residues can be determined in a few days using a relatively small sample (0.25 μmole) of protein or large peptide. Some technical problems, such as the identification of one or two residues, are yet to be overcome, but the method has a great potential for the compilation of sequences. The specific

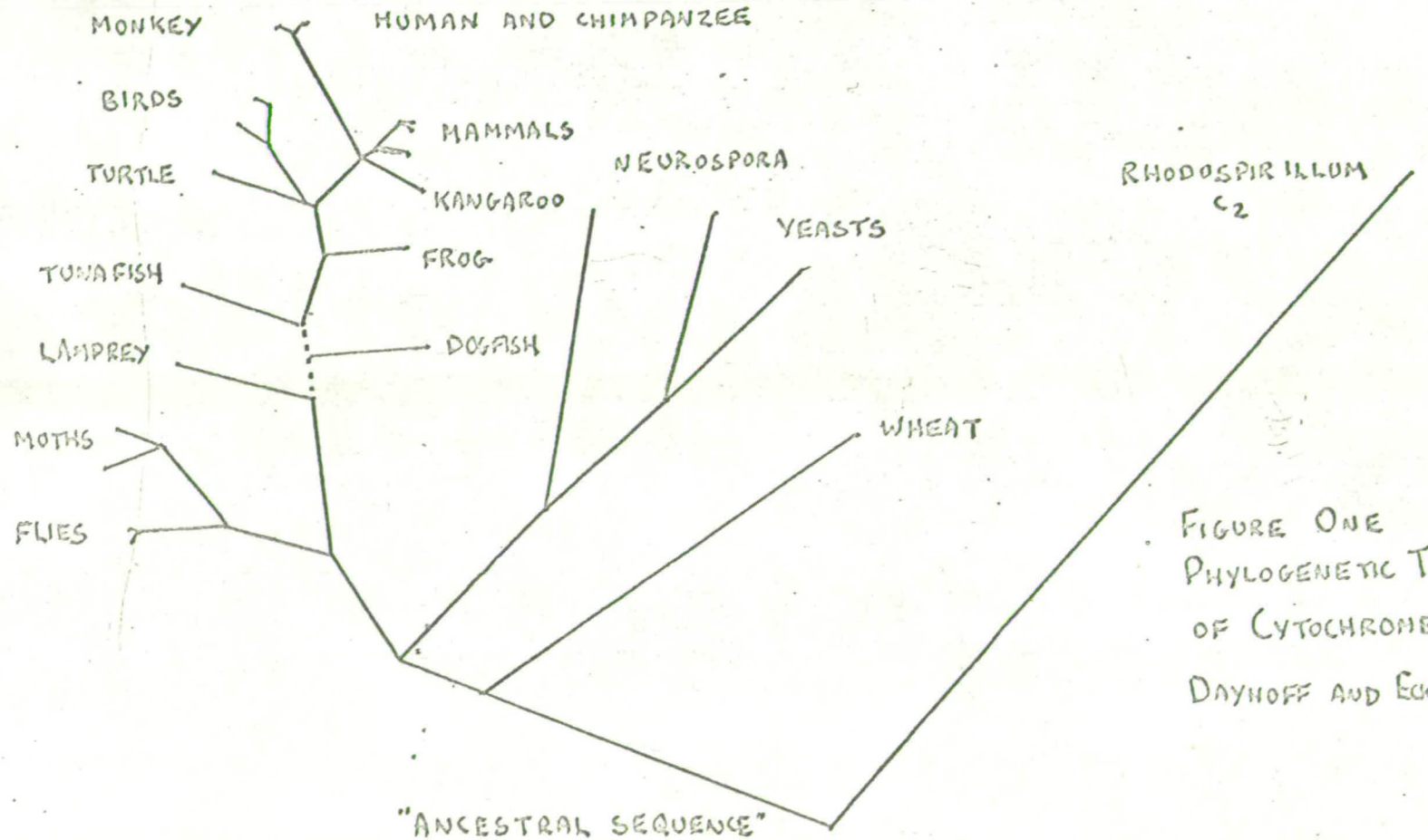


FIGURE ONE
PHYLOGENETIC TREE
OF CYTOCHRONE C.
DAYHOFF AND ECK 1971.

generation and fractionation of a few large peptide fragments will permit the complete sequence determination for large proteins using this technique. The traditional methods will probably often complement automatic sequencing, particularly in finding "overlapping" peptides.

Some peptide sequences have been determined by mass spectroscopy (Morris et al., 1971). The spectrum of a volatile derivative of a peptide reveals a series of molecular ions of decreasing mass-number, corresponding to a loss of amino-acid residues from the N-terminus. The successive decrements in mass-number are characteristic of the amino-acids lost. Simple mixtures of peptides can be simultaneously sequenced in many cases, as the relative intensities of the peptide spectra are different. This technique may ultimately reduce considerably the amount of peptide purification needed in protein sequencing.

Protein sequencing is now a relatively straightforward technique and sequences of functionally homologous proteins from various species have been elucidated. Comparison of these homologous sequences for any two organisms often reveals some identical and some different residues at corresponding positions, frequently with deletions or insertions of residues. Superficially, for each different protein, the number of differences between the sequences is directly proportional to the elapsed time since the corresponding organisms had a common ancestor, as deduced from geological and morphological evidence. Thus a phylogenetic tree can be constructed using the sequences of, for example, cytochrome C (Figure 1.). Simple computation of differences gives an approximate but useful tree. There has been developed a more sophisticated method which

"retraces the evolutionary pathway in reverse" (Dayhoff and Eck, 1971). It starts by comparing each position for a pair of very closely related sequences and deciding on an "ancestral" residue at each position, using the genetic code to predict the most likely mutations. This procedure is carried out for all the protein sequences available, using a computer, and arrives at an "ancestral sequence" for the protein. The method makes some provision for mutational reversion and thus can claim to produce a quantitatively more accurate phylogenetic tree.

There are in all series of sequences examined in this way some invarient residues which seem to be essential to the function of the protein; prosthetic-group binding, specific interactions between residues and proline residues which delineate regions of helical structure are typical examples. Indeed, by definition, homologous proteins must have "conserved" sequences. At some positions, there appear to be "conservative" substitutions; there is a requirement for an acidic or a hydrophobic group in some interaction, for example, or for a small residue at a point where peptide chains interact in the tertiary structure, and any one of the relevant few amino-acids can fill this requirement. At the other positions, there are a wide range of "non-conservative" substitutions. For cytochrome C, there has been a relatively slow and constant rate of amino-acid substitution at these positions. This may indicate that there has been effectively no selection acting upon the evolution of the sequence. Certainly, strong selective pressures would be expected to give rise to discontinuous evolution. From the teleological point of view, cytochrome C, as an intermediary between the electron transport system and molecular oxygen, might be

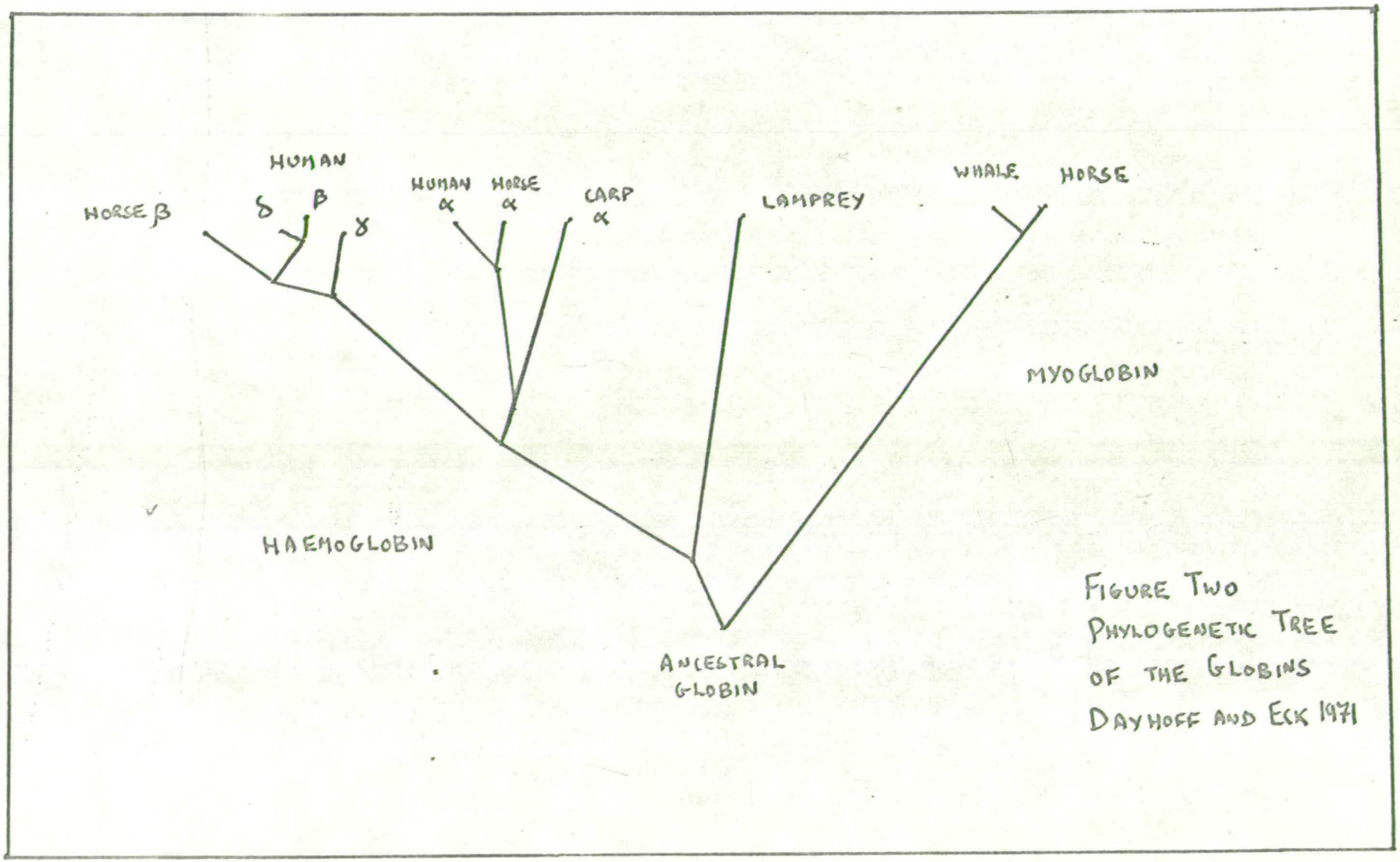


FIGURE TWO
PHYLOGENETIC TREE
OF THE GLOBINS
DAYHOFF AND ECK 1971

expected to fulfil a fundamental and relatively unchanging role.

The globins are a type of haem protein involved in oxygen transport. During the course of the evolution of these proteins, gene duplication is presumed to have given rise to new sequences which have evolved to fulfil slightly different functions. The phylogenetic tree of the globins is shown in Figure 2; unlike the cytochrome C tree it does not exactly parallel the phylogenetic tree of the species from which the proteins originate because some of the branching points represent speciation and some represent gene duplication.

Myoglobin and lamprey haemoglobin are monomeric haem proteins of molecular weight 17,000 daltons. Their sequences clearly have a common ancestor. The various haemoglobins of higher organisms are tetramers, the subunits of which have a similar molecular weight and which show sequence homology with each other and with myoglobin and lamprey haemoglobin. Many organisms have four or more types of haemoglobin subunit, as well as myoglobin. The sequences of these proteins have evolved independently from the point of divergence; human α-haemoglobin and myoglobin are more different than human and horse α-haemoglobins (Jukes, 1966). The rate of amino-acid substitution in α-haemoglobin is apparently constant and slightly less than the corresponding rate in β-haemoglobin, but several times higher than in cytochrome C. When sequences for frog β-haemoglobins were determined, the number of "invariant" residues fell from 70 to 54 (Chauvet and Acher, 1972). When all globins are considered, there are only 9 invariant residues, all of which are involved in haem binding (Jukes, 1966). This may reflect the effect of random, non-selected mutation and substitution in a more

"tolerant" structure than cytochrome C. The existence of several, functionally distinct types of globin in a single organism indicates that there has been, and probably still is, scope for the selective evolution of functionally more efficient haemoglobin sequences. Mutation and selection must always occur; their effects on primitive and on highly-developed sequences are different.

Sequence data of the cytochrome C type is most useful in taxonomy and phylogeny. Sequence data of the globin type is perhaps more useful in determining taxonomic and phylogenetic fine structure and in the study of the interrelationships between structure, function, mutation and natural selection. Amino-acid sequence data and the three-dimensional structure of α-haemoglobin have permitted an excellent analysis of the effects of amino-acid substitution on the structure and function of the protein and these effects have been correlated with the attendent clinical symptoms (Perutz and Lehmann, 1968). The bacterial penicillinases, to which the present work relates, will probably be more like the globins than cytochrome C in this respect.

In addition to the sources of genetic variability which make evolution possible in eucaryotes, there is in bacteria considerable scope for the transfer of genetic material between organisms (Hedges, 1972); thus there is the possibility that basic phylogenetic relationships may be overlaid and perhaps obscured by patterns based on the acquisition of characters or groups of characters from a "central pool". The occurrence and the amino-acid sequences of different respiratory proteins in various pseudomonads supports this view (Ambler, 1971).

The discovery of a penicillinase was only a few months after

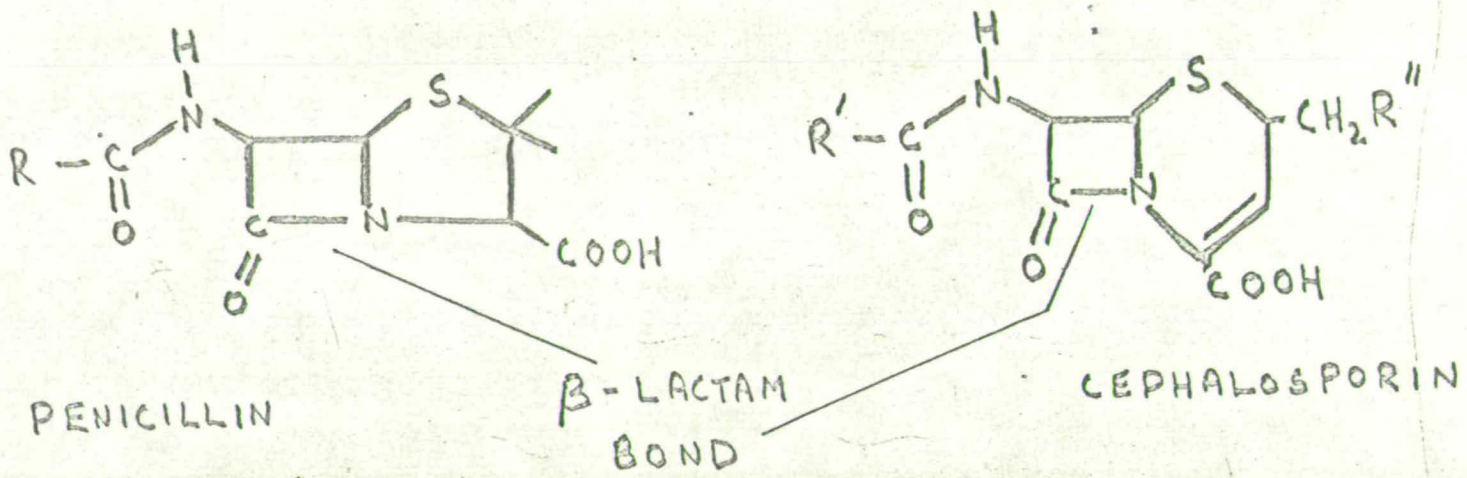


FIGURE 3.

the introduction of penicillin as a therapeutic agent (Pollock, 1971). Since then, "penicillinases" have been discovered in an exceedingly wide range of bacteria. The mode of penicillin hydrolysis in the original isolate is unknown, but most of those since discovered have been found to hydrolyse the β-lactam bond in penicillins and cephalosporins (Figure 3). These enzymes are now commonly referred to as β-lactamases (penicillin amido-β-lactam hydrolase, EC. 3.5.2.6.), to prevent confusion with penicillin amidase, which hydrolyses the amide bond between the penicillin nucleus and its side-chain. A representative selection of those β-lactamases which have been characterised, together with some salient features and the original references, is shown in Table 1.

The inducible, β-lactamase producing, Gram-positive bacteria readily yield constitutive mutants which produce the enzyme as up to 1% of the dry weight of the organisms, much of this being released into the culture supernatant. Thus, large-scale purification of these enzymes in the laboratory is relatively straightforward and hence they have been extensively and reliably characterised. The Gram-positive β-lactamases are relatively alike. There is an immunological cross-reaction between enzymes from Bacillus cereus and Bacillus licheniformis which argues for considerable sequence homology (Pollock, 1968). This homology has recently been directly demonstrated (Thatcher, 1972). The B.licheniformis and Staphylococcus aureus enzymes, which have no immunological cross-reaction, are homologous in 40% of their sequences (Ambler and Meadway, 1969). Other physical and chemical properties of these enzymes are very similar. The available evidence strongly suggests that the Gram-positive β-lactamases have evolved from a common

	SOURCE OF PENICILLINASE	INDUCIBILITY	PRODUCTION OF ENZYME % OF WET WEIGHT CELLS	LOCATION OF GENE	MOLECULAR WEIGHT x 10^{-4} DALTONS	RELATIVE ACTIVITIES WITH SUBSTRATES: BENZYLPENICILLIN	AMPICILLIN	6-AMINO-PENICILLANIC ACID	CEPHALOSPORIN C	REFERENCES
1.	BACILLUS LICHENIFORMIS 749	+	1·2	C	3·0	100	70	5	0·3	POLLOCK, 1965
2.	BACILLUS CEREUS 569/H I	+	1·1	C	3·1	100	120	40	+	KOGUT ET AL. 1956
	BACILLUS CEREUS 569/H II	+		C	2·2	100	64	–	80	KUWABARA AND ABRAHAM, 1969
3.	STAPHYLOCOCCUS AUREUS	+	0·9	P	3·0	100	ND	10	0·5	RICHMOND, 1963, 1965
4.	ESCHERICHIA COLI TEM	–	0·1	P	2·2	100	162	ND	125	DATTA AND RICHMOND, 1965[2] PRESENT WORK
5.	E. COLI R GN 14	–	ND	P	2·1	ND				YAMAGISHI ET AL., 1969
6	E. COLI R GN 238	–	ND	P	2·8[1]	ND				YAMAGISHI ET AL., 1969
7.	E. COLI R1	–	0·1	P	2·2	100	150	ND	50	LINDQVIST AND NORDSTROM, 1970
8.	A. CLOACAE P53	–	ND	P	ND	100	120	ND	150	SMITH, 1963.[2]
9.	A. CLOACAE P99	–	ND	P	ND	100	0	ND	8000	GOLDNER ET AL.,[2] 1968
10.	PSEUDOMONAS AERUGINOSA[3]	–	ND	P	ND	100	160	ND	125	SYKES AND RICHMOND, 1970
11.	SALMONELLA TYPHIMURIUM	–	ND	P	3·0	100	100	68	8	NEU AND WINSCHELL, 1970
12.	E. COLI K 12	–	0·1	C	2·9	ND				LINDSTRÖM ET AL, 1970
13.	E. COLI DH. 131	–	ND	C	3·0	100	100	74	8	NEU AND WINSCHELL, 1970

[1] AVERAGE VALUE

[2] QUOTED BY JACK AND RICHMOND, 1970.

[3] INDISTINGUISHABLE FROM E. COLI TEM β-LACTAMASE USING ENZYMOLOGICAL AND IMMUNOLOGICAL CRITERIA

ND NOT DETERMINED.

Table 1

ancestral protein.

The Gram-negative β-lactamase producers are usually non-inducible and are characterised by a much lower proportion of the enzyme, which is normally cell-bound. Purification is correspondingly more difficult and it is only recently that an accumulation of the properties of purified enzymes from these sources has been possible. The majority of Gram-negative β-lactamases are specified by genes which are carried on transferable episomes called resistance transfer factors (RTF). The RTF of one β-lactamase-producing strain of Salmonella typhimurium can be transferred to strains of Escherichia coli, Shigella sonnei, Serratia marcescens and Proteus mirabilis. Each recipient strain subsequently produces an enzyme indistinguishable from that produced by the donor strain (Neu and Winschell, 1970; see Table 1, examples 4 and 10). Examples 4-11 in Table 1, and many of those in a recent, extensive comparative study*, suggest that there may be several varieties of enzyme of about the same molecular weight but with different specificities with respect to the various penicillins and cephalosporins. These enzymes may well have extensive sequence homology. The interchangeability of RTFs and the possibility of hybridisation due to genetic recombination between RTFs implies that no β-lactamase in this group can be characteristic of a particular species or strain and that the enzymes may exhibit a continuum of variation in amino-acid sequence.

There are few apparent similarities between β-lactamases of this type and those from Gram-positive bacteria but it must be admitted that the differences are mainly in the physiology of enzyme production and that there is very little comparative chemical

* Jack and Richmond, 1970.

data. The β-lactamase from E.coli TEM was chosen as being representative of this group of Gram-negative β-lactamases for an amino-acid sequence determination, to establish whether or not the two types of enzyme have a common ancestor. The limitations discussed above preclude any conclusive bacterial phylogeny or taxonomy based upon β-lactamase sequences.

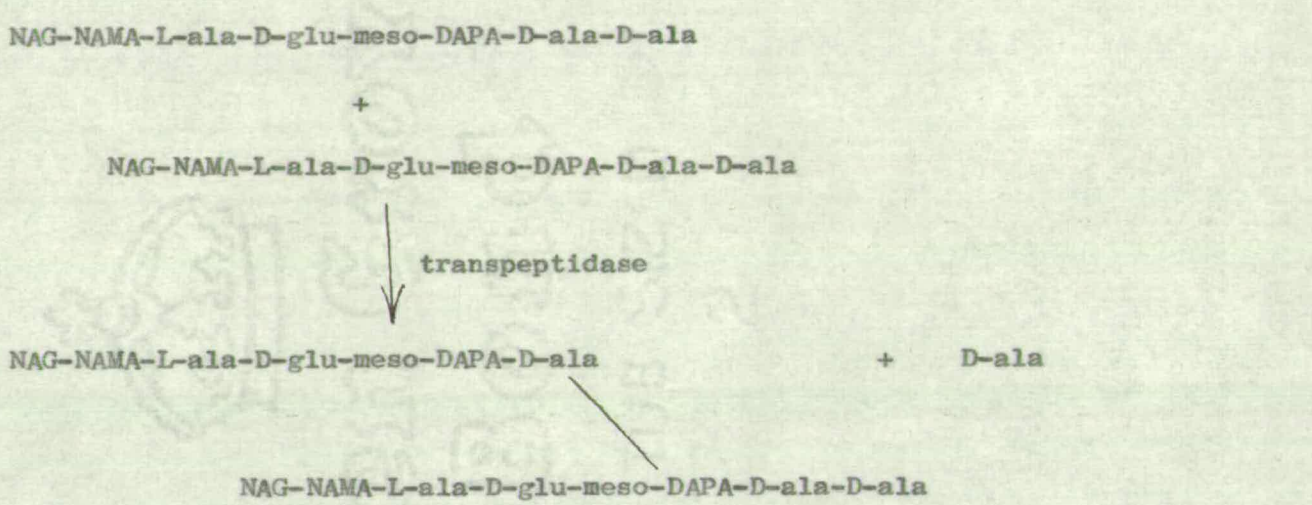
There are some β-lactamases, from Gram-negative bacteria, which have molecular weights of around 30,000 daltons and which are specified by chromosomal genes. (Examples 12 and 12 in Table 1). In these and other properties, they show some similarities with Gram-positive β-lactamases. The apparent lack of homology between RTFs and bacterial chromosomes (Meynell et al., 1968) suggests that this type of β-lactamase may be relatively unrelated to the RTF-mediated β-lactamases.

Penicillins and cephalosporins exert their antibiotic effect because they are structural analogues of an intermediate in the synthesis of bacterial cell walls; inhibition of cell wall synthesis leads to lysis and death (Park, 1966). The sensitive enzyme is thought to be the transpeptidase which cleaves the D-ala-D-ala bond in the cell wall mucopeptide (Strominger et al., 1971; see Figure 4).

The coexistence of penicillin-sensitive bacteria and penicillin-producing microfungi in various milieu has been demonstrated (Pollock, 1967, 1971; Smith and Marples, 1964). Furthermore, β-lactamase has been shown to confer resistance to penicillin at the concentrations of the antibiotic encountered in the environment (Pollock, 1972). β-lactamase-producing strains of B.cereus are more viable in the presence of penicillin-producing Penicillium than strains which do not produce the enzyme (Hill, 1972).

Figure 4. Synthesis of Cell Wall Glycopeptide: The Transpeptidase Step in E.coli

(Strominger et al., 1971)



NAG : N-acetylglucosamine NAMA : N-acetylmuramic acid DAPA : Diaminopimelic acid

β-lactamase has not been found in organisms which do not have penicillin-sensitive cell walls. We can reasonably assume that there has been, and still is, a considerable selective pressure for the maintenance and evolution of β-lactamase.

An attractive hypothesis is that duplication of a gene such as that coding for the cell-wall transpeptidase, followed by mutation and/or recombination in the redundant gene, led to the evolution of β-lactamase (Pollock, 1967). The various bacterial cell wall structures so far studied are basically very similar but have distinct and characteristic differences. Various β-lactamases may have originated independently, from their respective cell-wall synthesising enzymes and may thus be considerably different from each other. The underlying similarities in cell wall structure argue, however, for common ancestry for their synthetic enzymes, and thus for a common, though distant, ancestor of all β-lactamases.

So far, the emphasis in this discussion has been on structure and the evolution of structure. Biological structure, however, is ultimately functional. It has been claimed (Saz, 1970) that β-lactamase activity is but an incidental function of a protein otherwise concerned in bacterial metabolism. This "real function" has yet to be demonstrated and β-lactamase-deficient mutants are normally viable (Pollock, 1971). The reasonable function of β-lactamase is the detoxification of β-lactam antibiotics.

Discussion of function brings us naturally to the question of mechanism. Quite apart from their intrinsic value, studies of the mechanism of β-lactamase may clarify evolutionary relationships. The differences in chemical, physical and enzymological properties of β-lactamases are such that the possibility of more than one

mechanism is not excluded. Certainly, the β-lactamase II of B.cereus (Example 2 in Table 1), which requires zinc ions for activity, must be considerably different in this respect from other β-lactamases. The "modified peptidase" theory of the origin of β-lactamase mentioned above is perhaps even more intriguing in view of the fact that carboxypeptidase A has a similar requirement for zinc ions (Hartsuck and Lipscomb, 1971).

Studies on the structure and function of enzymes can be subdivided as follows:

(1) Characterisation of the reaction and determination of the kinetic parameters under various conditions.

(2) Chemical modification of residues and the effect on activity. Structural studies identifying particular residues. The amino-acid sequence.

(3) X-ray diffraction to determine the three-dimensional structure of the enzyme.

The occurrence and stoichiometry of many enzyme reactions was demonstrated in the late 19th century and, early in this century, a simple and effective theory of enzyme action, which could be applied to the kinetics of many enzyme reactions, was developed (reviewed by Dixon and Webb, 1964).

The concept of a specific site on the enzyme for the substrate is inherent in the kinetic theories of enzyme reactions and can be traced back to Fischer's famous "lock and key" hypothesis (Fischer, 1894). Identification of the amino-acid residues involved at this site awaited the development of techniques in enzyme purification and amino-acid analysis and, perhaps more significantly, an acceptance of the concept of proteins as polypeptides of definite,

discrete and characteristic structure. The identification of serine at the active site of trypsin, and the extensive studies of Weil and co-workers on the photo-oxidation of enzymes, were early examples in this field (Jansen and Balls, 1952; Weil et al., 1951). Fairly extensive chemical modification studies have accompanied many amino-acid sequence determinations, the comparatively recent work on staphylococcal nuclease being an excellent example (Anfinsen et al., 1971). Koshland has demonstrated the importance of a kinetic correlation between the modification of a tentative "active-site residue" and the loss of activity of the enzyme (Ray and Koshland, 1960).

On the basis of the kinetics and a knowledge of the residues involved in the active site, mechanisms of enzyme action can and have been proposed. The determination of the three-dimensional structure of the enzyme is essential for the confirmation and refinement of these mechanisms. This has been accomplished for several hydrolytic enzymes such as lysozyme, ribonuclease and chymotrypsin.

Despite this mechanistic knowledge, the precise origins of the rate enhancement caused by enzymes are still uncertain and much debated (Hoare, 1972; Gutfreund and Knowles, 1967). Research in this area is mainly confined to model systems in which the precise orientation of reactant and catalytic moieties may approximate to an enzyme-substrate relationship.

In the main, β-lactamase enzymology has been restricted to the determination of the relative rates of hydrolysis of the various penicillins and cephalosporins, and of the relevant substrate and inhibition constants (Citri, 1971). The concept of "conformative

response" represents an attempt to further exploit the wide range of available substrates. The effects of various inhibitory procedures, such as photo-oxidation and iodination, on B.cereus β-lactamase, measured with and without substrate present, are compared. The β-lactam nucleus appears to stabilise the enzyme, and this effect is enhanced by adding a small side-chain. Bulky side-chains cause increased inhibition (Citri and Zyk, 1965).

In the field of chemical modification, the specific nitrations of single tyrosine residues in B.licheniformis and S.aureus β-lactamases respectively, with the resultant partial losses in activity, were the first steps towards an understanding of the mechanism of β-lactamase (Meadway, 1969b; R.P. Ambler, unpublished results). Very recently, the iodination of a single tyrosine residue in B-cereus β-lactamase, again with partial loss of activity, has been reported (Csanyi et al., 1971).

An interesting model system was investigated by Schwartz and co-workers. Starting from the observation that nucleophiles such as hydroxyl ion catalyse penicillin hydrolysis, they investigated the catalytic properties of a range of phenolic compounds in this reaction. The catecholamine derivative, 3,6-bis (dimethylaminomethyl) catechol (CDM) was found to be especially effective. The variation of activity with pH indicated that CDM^{+} was the effective ionic species and comparison with other isomers of CDM showed that the relative positions of the various groups was important. They also found some evidence for a penicilloate ester of CDM as a reaction intermediate.

The proposed mechanism of penicillin hydrolysis catalysed by CDM is shown in Figure 5. One of the positively-charged nitrogens

1. PENICILLIN.

CDM.

2. TETRAHEDRAL INTERMEDIATE

3.

4. PENICILLOATE ESTER OF CDM

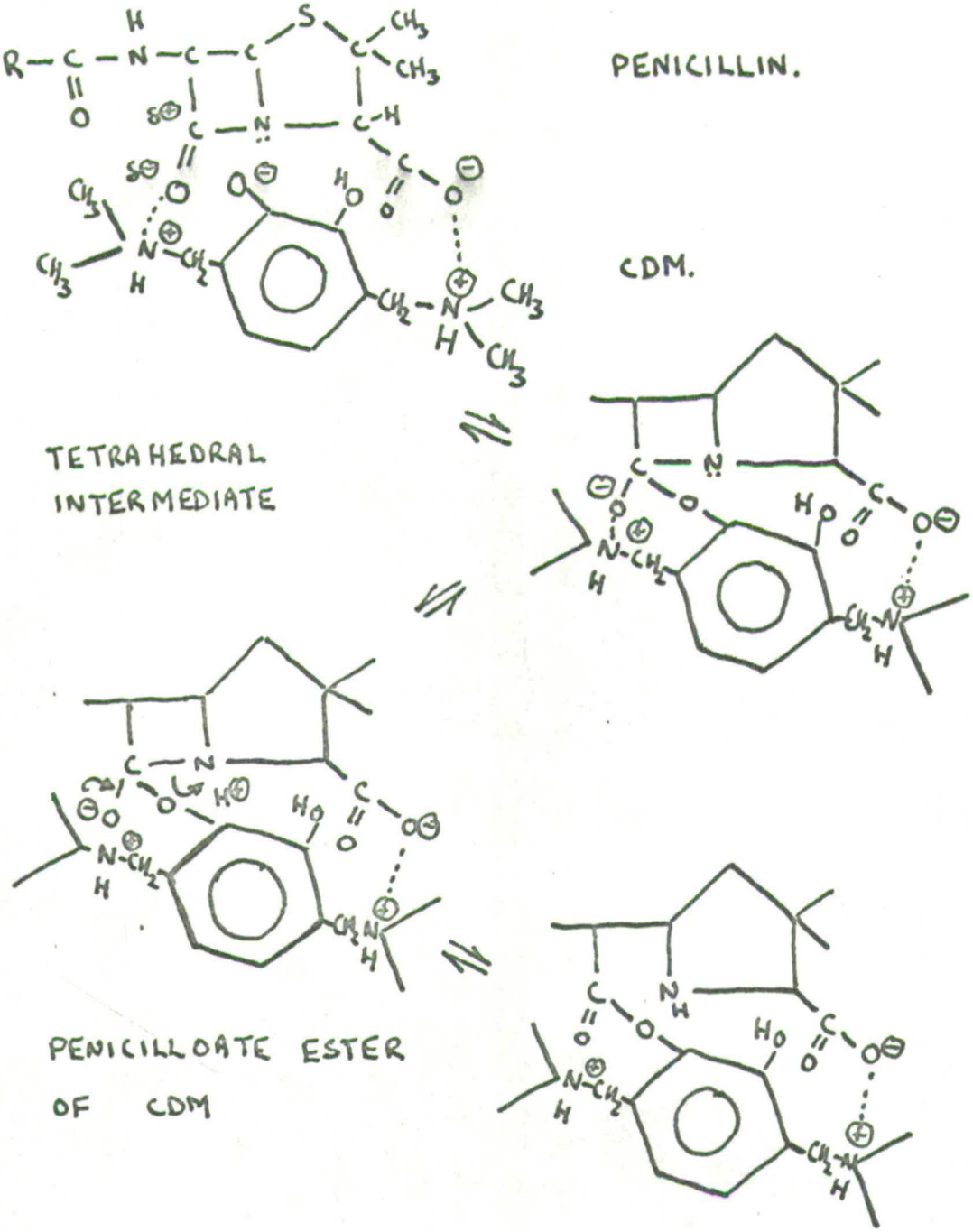


FIGURE 5.

CATALYSIS OF PENICILLIN HYDROLYSIS BY CDM.

in CDM serves to bind the oxygen in the acid group of penicillin, whilst the other polarises the β-lactam carbonyl bond, thus promoting nucleophilic attack at the carbonyl carbon atom. An intermediate compound is formed, stabilised by two electrostatic interactions. This gives rise to the penicilloate ester of CDM. Hydrolysis of the ester gives penicilloic acid and free CDM. The functional groups in CDM could well be present in the side-chains of amino-acids involved in the active site of β-lactamase (Schwartz, 1965; Schwarts and Pflug, 1967; Kinget and Schwartz, 1968).

The following thesis describes experiments on the relationship between structure and activity in Escherichia coli TEM β-lactamase carried out in conjunction with work on the primary structure of this enzyme.

The chapters describing the purification and properties of the enzyme, the N- and C-terminal regions, and the enzymology and chemical modification, each have their own sections describing the methods used therein. The relevant methodology in protein sequence determination is described in Chapter IV, which precedes the chapters describing the results of the work on the primary structure.

Table 2 lists the abbreviations used in this thesis: the recommendations contained in Instructions to Authors of the Biochemical Journal (1971) have been followed. Table 3 lists the three-letter and single-letter codes for the amino-acids, in accordance with the IUPAC/IUB Commission on Nomenclature (1967, 1969).

Table 2. List of Abbreviations

BAWP	oButan-1-ol:acetic acid:water:pyridine::15:3:12:10
CM-	Carboxymethyl-
CNBr	Cyanogen Bromide
CPA	Carboxypeptidase A
CPB	Carboxypeptidase B
DEAE-	Diethylaminoethyl-
DFP	Diisopropylfluorophosphate
Dnp-	2,4-dinitrophenyl-
Dns- (dansyl-)	1-dimethylaminonaphthalene-5-sulphonyl-
DPCC-trypsin	Trypsin treated with diphenylcarbamyl chloride
ETPA	exo-cis-3,6-endoxo-Δ^4-tetrahydrophthalic anhydride
ETP-	the radical of ETPA
FDNB	1-fluoro-2,4-dinitrobenzene
IAA	Iodoacetic acid
K_m	The Michaelis substrate constant
LVIF	Unresolved Dns-leu/val/ile/phe
m	Electrophoretic mobility at pH 6.5
m'	Electrophoretic mobility at pH 3.5
NEM	N-ethyl morpholine
PEAW	Light petroleum (b.p 40°-60°):acetic acid:water::10:9:1
PITC	phenylisothiocyanate
PTC-	phenylthiocarbamyl-
PTH-	phenylthiohydantoin-
RTF	Resistance transfer factor
R_X	Chromatographic mobility relative to XCFF

Table 2. - Continued

SBTI	Soya bean trypsin inhibitor
SDS	Sodium dodecyl sulphate
SGA	Unresolved DNS ser/gly/ala
SPITC	4-sulphophenylisothiocyanate
TCA	Trichloracetic acid
TFA	Trifluoroacetic acid
TNM	Tetranitromethane
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
V	Maximum enzymic reaction velocity (Molecular activity)
XCFF	Xylene cyanol FF

Solutions are specified by a weight of solid or a volume of liquid dissolved in a volume of solvent, unless otherwise noted.

Table 3. Abbreviations of Amino-Acids

Amino-acid	Three-letter code	One-letter code
lysine	lys	k
histidine	his	h
arginine	arg	r
cysteic acid	$cySO_3H$	c
aspartic acid	asp	d
asparigine	asn	n
aspartic acid or asparigine	asx	b
methionine sulphone	mes	m
threonine	thr	t
serine	ser	s
glutamic acid	glu	e
glutamine	gln	q
glutamic acid or glutamine	glx	z
proline	pro	p
glycine	gly	g
alanine	ala	a
cysteine	cys	c
valine	val	v
methionine	met	m
isoleucine	ile	i
leucine	leu	l
tyrosine	tyr	y
phenylalanine	phe	f
tryptophan	trp	w
homoserine	hsr	-
homoserine lactone	hsl	-
3-nitrotyrosine	$tyrNO_2$	-
carboxymethylhistidine	CM-his	-
carboxymethylcysteine	CM-cys	-

Amino-acids which have been sequenced are foined by hyphens:

asp-glu-arg-asn-arg-

Amino-acids which have not been sequenced are placed in brackets:

asp-glu-arg-asn-arg-(glu,ile,ala)

CHAPTER II.

PURIFICATION AND PROPERTIES OF *E.COLI* β-LACTAMASE

The main sources of general laboratory chemicals were Fisons Scientific Apparatus and the British Drug Houses Laboratory Chemicals Division. The latter was also the source of standard DNS-amino-acids and of most stains and dyes. Sodium benzylpenicillin was a gift from Glaxo Laboratories. Other sources are quoted in the relevant methods. Buffers were generally made up according to tables in "Data for Biochemical Research" p.205 (ed. Dawson *et al.*, 1959).

The β-lactamase was prepared from the *E.coli* strain W3310, which carried the TEM R-factor and which was a gift from Professor M.H. Richmond (Dept. of Bacteriology, University of Bristol) to Dr. J.W. Melling, who carried out the large-scale bacterial culture and the preliminary purification of the enzyme, at the Microbiological Research Establishment, Porton Down.

Assay Techniques

A spectrophotometric variant of the Perret β-lactamase assay was routinely used (Perret, 1954; Sherratt, 1969; Sherratt and Collins, in press). The following solutions were used:

(i) 2.4 mg per ml sodium benzylpenicillin in 0.1 M sodium phosphate buffer pH 7.0.

(ii) 40.8 g of iodine and 200 g potassium iodide in 1 litre of water. This "stock solution" was diluted 20-fold with 0.075 M ammonium acetate buffer pH 3.6.

The penicillin solution was incubated in 2.5 ml aliquots at 30°C. A sample (V ml) of the β-lactamase solution was added to

one aliquot and incubated for a convenient time (t min), after which 5.0 ml of dilute iodine was added, with mixing. A reaction blank was made by adding 5.0 ml of dilute iodine to a second aliquot, followed by V ml of β-lactamase solution. The samples were incubated at room temperature for 10 min and the difference in extinction at 499 nm (E) was measured.

The β-lactamase activity in units per ml was given by:

$$E \times \frac{1}{V} \times (\text{total volume}) \times \frac{60}{t}$$

$$\text{total volume} = (7.5 + V)$$

The unit is defined as the β-lactamase activity which will hydrolyse 1 μmole of penicillin per hour at 30°C (Pollock and Torriani, 1953). This unit has been widely used and will be used throughout the present work: in Appendix 1, the units comply with the recommendations of the IUB Enzyme Commission i.e. 1 International Unit of β-lactamase will hydrolyse 1 μmole of penicillin in 1 minute at 30°C.

The spectrophotometric-Perret assay is reproducible, sensitive and very convenient.

Michaelis parameters were determined using the Novick microiodometric assay (Novick, 1962). The following solutions were used:-

(i) 0.05 M sodium phosphate buffer pH 7.0.

(ii) 0.08 M iodine in 3.2 M potassium iodide.

(iii) A fresh, boiled 2% starch solution (Connaught).

(iv) 20 ml of this starch solution added to 180 ml of buffer and 0.30 ml of iodine solution.

(v) Solutions of sodium benzylpenicillin in buffer (0.03 to 0.6 mM).

To each of two 1.0 cm spectrophotometer cuvettes was added:

1.0 ml of starch/iodine solution

1.0 ml of penicillin solution

0.9 ml of buffer (1.0 ml in blank)

0.2 ml of starch solution.

The cuvettes were equilibrated at 30°C in a double-beam spectrophotometer (Beckman DB) and 0.1 ml of enzyme solution containing 0.1 to 0.2 units of activity (as defined in the previous section) was added to the cuvette in the "reference" position in the spectrophotometer. This caused an apparent increase in extinction at 620 nm which was followed for 5-10 min. The rate of penicillin hydrolysis (V) is given by:

$$V = \frac{E}{0.128 \times t} \quad \text{units per litre}$$

where E is the increase in extinction during the time t in hours.

In practice, an enzyme solution was diluted to about 0.5 U/ml and then further diluted so that at the highest substrate concentration (200 μM in the cuvette), complete decolourisation of trial reaction mixtures occurred in about 3 min. This enzyme solution could then be used in the assay with substrate concentrations between 10 and 200 μM: below this figure, the rate was not reproducible.

The omission of heat-killed enzyme solution from the reaction blank (Novick, 1962) was not critical.

In the original β-lactamase purification, assays were done at pH 5.9 (Datta and Richmond, 1965). In my experience, the activity is slightly higher at pH 7.0 and furthermore the enzyme is unstable if kept below pH 6.0 for any length of time.

The Lowry assay for protein was used in purification procedures

(Lowry et al., 1951). The following reagents were used:

(i) Reagent A (fresh daily): 1 ml of 2% sodium potassium tartrate, plus 1 ml 1% cupric sulphate pentahydrate in 100 ml 2% sodium carbonate in 0.1 M sodium hydroxide.

(ii) Reagent B (stable at 4°C): Folin-Ciocalteau reagent diluted two-fold.

(iii) 1 mg/ml bovine serum albumin.

The samples to be analysed were made up to 0.5 ml with water. A 0.5 ml water blank and standards containing 0.05-0.2 ml of the BSA solution were included. 3 ml of reagent A was added to each sample and the tubes were shaken and left at room temperature for 10 min. 0.3 ml of reagent B was added, with mixing. After 1 hr, the extinction at 750 nm was read. A calibration curve was made "in situ" with the standards.

A pure β-lactamase solution was estimated as containing 0.60 mg/ml using this assay. Amino-acid analysis of the same solution gave an estimate of 0.57 mg/ml, assuming a molecular weight of 2.1×10^4 daltons.

Chromatography and Gel Filtration

DEAE-cellulose (Whatman DE-52) was used for the ion-exchange chromatography of β-lactamase. This resin was supplied ready for use and about 70 g of dry resin sufficed for a column 20 cm x 2.5 cm. It was stirred for 10-20 min with 400 ml of buffer ten times more concentrated than that used for chromatography. When the resin had settled, this buffer was decanted off and the resin was washed in the same way with several aliquots of the chromatographic buffer. Used resin was pooled, washed with water and then stirred in 0.5 M HCl for 30 min and washed with water until at pH 4. It was then

stirred with 0.5 M NaOH for 30 min and washed with water until at pH 7. It could then be stored at 4°C, ready for further use. After several cycles, the resin was discarded.

Gel filtration was used as a purification technique. Sephadex G-75 (fine grade; Pharmacia) was swollen quickly by stirring the dry powder into a large volume of water and maintaining the slurry at 100°C for 4-5 hr. It was cooled and settled and resuspended several times in 0.04 M sodium phosphate buffer pH 7.0. Columns (Whatman and Pharmacia) were packed under gravity, with a pump (Perpex, LKB) limiting the flowrate to that required for gel filtration. Samples for gel filtration were mixed with a few drops of ϵ-Dnp-lysine solution which is strongly retarded by the gel and which indicates when the column is completely eluted.

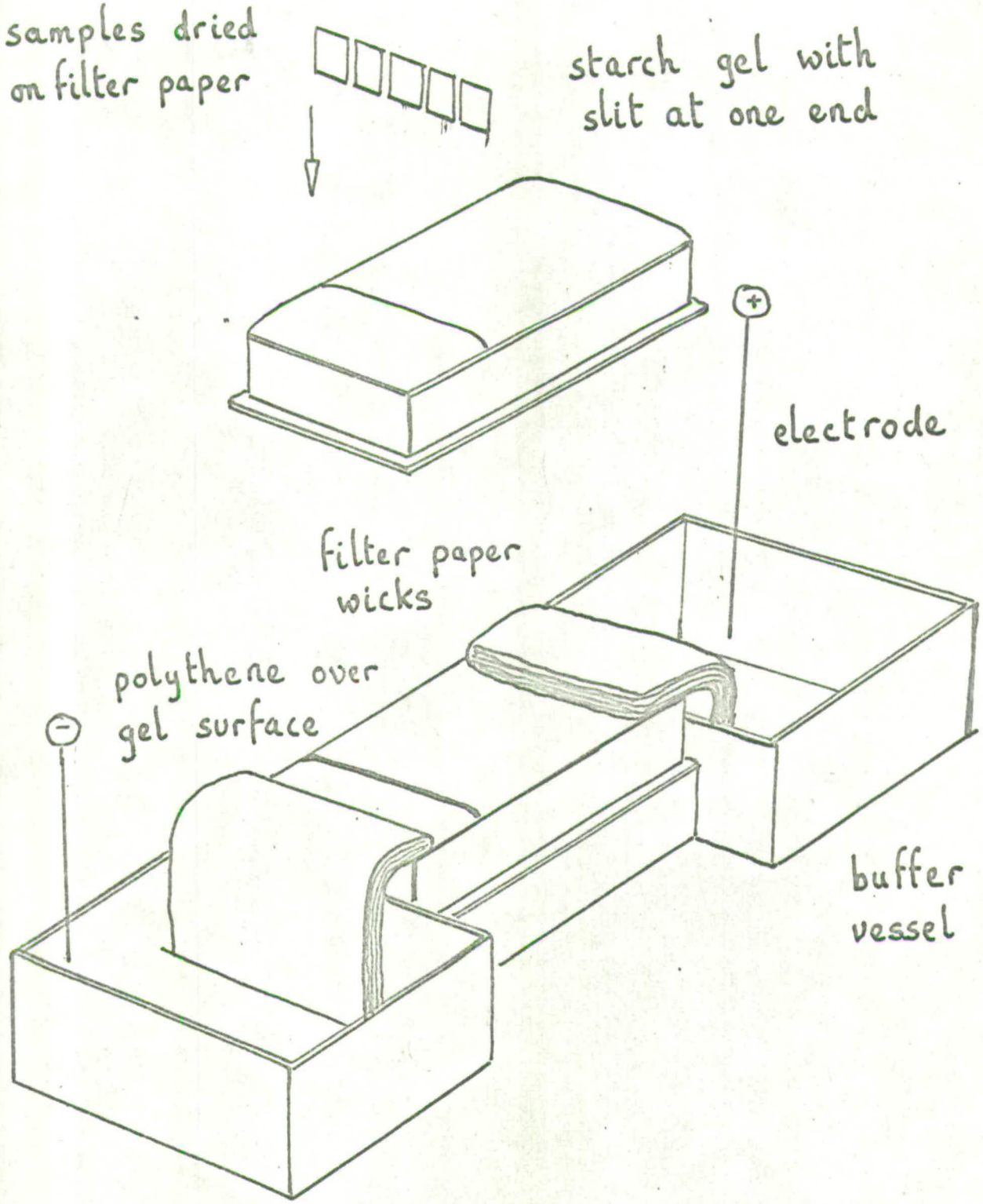
Column effluents were subjected to continuous spectrophotometric monitoring at wavelength 254 nm.

The purity of a sample of β-lactamase was confirmed in one case by gel filtration of a small sample on a small column of G-75 equilibrated with 50% formic acid.

Sephadex G-25 and G-15 could be swollen quickly, equilibrated with 0.1 M ammonia or 5% formic acid and used for desalting protein samples or for peptide fractionation.

The molecular weight of β-lactamase was estimated by the method of Andrews (1964). A column (88 cm x 2.5 cm) of Sephadex G-75 (superfine grade) in 0.04 M sodium phosphate buffer pH 7.0 was set up, and β-lactamase and various proteins of known molecular weight were used as samples. Their respective elution volumes were noted. Blue dextran (Pharmacia), a high molecular weight dextran coupled to a coloured dye, was used to determine the void volume (V_v) of the

FIGURE 6 STARCH GEL ELECTROPHORESIS



column. The logarithm of the molecular weight of a protein is proportional to V_e for the protein, where V_e = (elution volume of sample - V_v).

Gel Electrophoresis

Starch-gel electrophoresis was used routinely as a criterion of purity (Smithies, 1955). A slurry of 17 g hydrolysed starch (Connaught) in 170 ml of 0.03 M buffer (sodium borate pH 8.5 or potassium hydrogen phthalate pH 4.0) was made in a 1-litre round-bottomed flask. It was heated strongly to boiling-point and then de-gassed at a water pump and poured into a perspex tray fitted with a false bottom (see Figure 6). When cool, the gel was covered with a sheet of polythene and could be stored for a few days at $4^{\circ}C$.

Small rectangles of filter paper were soaked in protein solution and dried and then inserted in a slit cut 5 cm from one end of the gel. A "marker" of XCFF was applied in the same way. The electrophoresis was carried out at $4^{\circ}C$ with moist filter paper wicks (8 thicknesses) pressed on to each end of the gel and dipping into troughs of 0.3 M buffer. Electrodes in the troughs were used to apply a potential difference of 250 V, at which a current of ca 50 ma was passed through the gel. A 2 hr run caused the XCFF to move 6-7 cm towards the positive electrode.

The gel was cooled and sliced by inserting spacers under the false bottom and cutting through the protruding section with a fine wire.

A slice was stained for protein by soaking for a few minutes in a 0.5% solution of 1-naphthamidoblack in methanol:water:acetic acid (5:5:2 by volume) and then washed repeatedly in this solvent to

remove excess stain. Blue bands corresponding to protein appeared on a pale blue background.

β-lactamase activity was detected by spraying with a solution of 0.6 g sodium benzylpenicillin in 50 ml 0.1 M phosphate buffer pH 7.0, containing 2.5 ml of stock iodine solution from the spectrophotometric β-lactamase assay. Rapidly-cleared spots on a dark blue background indicated β-lactamase activity.

Electrophoresis in SDS-polyacrylamide gels was done by the method of Weber and Osborn (1969). The following solutions were required:

(i) Gel buffer consisted of 7.8 g NaH_2PO_4 H_2O, 38.6 g $Na_2HPO_4 \cdot 7H_2O$ and 2.0 g SDS per litre. This was diluted with 50% water to make electrophoresis buffer.

(ii) 22.2 g acrylamide (Eastman recrystallised from chloroform) and 0.6 g N,N'-methylene-bis-acrylamide dissolved in water and made up to 100 ml. Filtered and stored in a dark bottle at 4°C.

(iii) 15 mg per ml ammonium persulphate (fresh).

(iv) 0.05% Bromophenol blue.

(v) The staining solution was made by dissolving 1.25 g Coomassie brilliant blue in 500 ml of methanol:water: acetic acid (225:225:50 by volume), and filtering.

(vi) The destaining solution consisted of methanol:water: acetic acid (50:875:75 by volume).

10 ml of gel buffer was de-gassed and mixed with 9 ml of acrylamide soln. and again de-gassed. Then 1 ml of fresh ammonium persulphate soln. was added together with 0.045 ml of N,N,N',N'-tetramethylethylenediamine (Eastman) and mixed well. Six acid-cleaned glass gel tubes (8 cm x 0.6 cm internal diameter, open at

both ends), each standing vertically with one end embedded in a block of Plasticene, were filled to about 1 cm from the top with the mixture and a few drops of water were added to cover the top of the gel. After 30 min the gels had hardened and the water was removed.

Protein solutions were pre-incubated for 2 hr in gel buffer containing 1% 2-mercaptoethanol. (0.05 mg protein in 100 buffer). To each gel was applied a mixture of 3 λ Bromophenol blue soln., 50 λ glycerol, 5 λ 2-mercaptoethanol and 50 λ protein solution. The tubes were placed in the apparatus (Shandon) and electrophoresis buffer was carefully layered on top. The electrode compartments were filled with buffer and the electrophoresis was carried out at 8 ma per gel for 4 hr at 4°C.

The gels were removed from the tubes by squirting water from a syringe between the gel and the wall of the tube and then applying pressure with compressed air. The lengths of the gels and the distances of migration of the dye were measured. The gels were stained in test-tubes for 3-4 hr, rinsed with distilled water and destained with successive batches of solvent over a period of several hours. The lengths of the gels and the distance of migration of the protein band(s) were measured. The mobility of a protein, relative to the dye and corrected for swelling during staining, was given by:

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}$$

Weber and Osborn have shown that under certain conditions, the mobility of a protein is directly proportional to the logarithm of its molecular weight.

Analytical Methods

The experimental procedures relating to automatic amino-acid analysis are described in Chapter IV.

Carbohydrate was determined by the method of Devor (1948). 0.4 g of 1-naphthol was dissolved in 100 ml H_2SO_4 and allowed to stand in the dark for several hours. Standards containing up to 0.1 mg of glucose in 1.0 ml of water were prepared, and 1.0 ml of 0.2 M ammonium acetate pH 4.0 was added to each. β-lactamase was dissolved in 3.5 ml 0.2 M acetate. Two 1.0 ml samples were made up to 2.0 ml with water and a third sample was analysed for amino-acids. 5.0 ml of the reagent was added to each sample and the samples were placed in a boiling water bath for 10 min. They were then cooled and the extinction at 575 nm, relative to a reagent blank, was measured. A standard curve was constructed and the carbohydrate content of the protein was determined.

Tryptophan determination was by the method of Spies and Chambers (1948). Standards of up to 0.7 μmole tryptophan and a protein sample of about 0.2 μmole were dissolved in 0.5 ml water and 4 ml of 65% H_2SO_4 and 0.5 ml of 3% dimethylaminobenzaldehyde in 1 M - H_2SO_4 were added to each sample. After mixing, they were incubated in the dark at room temperature for 1 hr. Then 0.05 ml of 0.04% sodium nitrite was added to each and, after mixing, the samples were incubated, as before, for 30 min. The extinction at 590 nm was measured relative to a reagent blank and the tryptophan content of the protein sample derived from the standard curve.

Cysteine was determined using iodo-acetic acid (Gurd, 1967). Approximately 2 mg of β-lactamase was dissolved in 0.5 ml of 0.3 M ammonium carbonate pH 8.3 in 6 M urea. 0.2 ml of this solution

was mixed with an equal volume of the same buffer containing 0.1 mmole (11 mg) of IAA, and incubated at room temperature for 2 hr. The sample, and 0.2 ml of the original protein solution, were each desalted on a small Sephadex G-15 column in 5% formic acid, and freeze-dried. Each sample was analysed for amino acids.

Purification of E.coli B-lactamase

This part of the work is concisely described in Appendix I, together with the details of the bacterial culture and the preliminary purification carried out by Dr. Melling.

Batches of impure β-lactamase containing up to 2×10^8 units were freeze-dried and transported by air to Edinburgh; prolonged storage at $4^{\circ}C$ in this state was not deleterious to enzymic activity.

A typical batch of 10^8 units was dissolved in 10.0 ml of 0.04 M sodium phosphate buffer pH 7.0. Any slight precipitation was removed by centrifugation. If the earlier dialysis was not exhaustive, this solution was very viscous, there was more precipitation and some loss of activity accurred during these processes. This solution was applied to a column of Sephadex G-75 (90 x 6 cm; equilibrated with 0.04 M phosphate pH 7.0; flow rate 100 ml/hr; 8.0 ml fractions). The pooled fractions from this column were dialysed and adjusted to pH 7.0 and conductivity about 220 μsiemens with 0.1 M phosphate pH 7.0.

This solution was passed through another DE-52 column (10 x 2.5 cm; equilibrated with 0.002 M phosphate pH 7.0). The column was washed with 150 ml 0.008 M buffer and then with a linear gradient consisting of 250 ml of 0.008 M buffer and 250 ml of 0.016 M buffer. 6.0 ml fractions were collected at a flow rate of 50 ml/hr. These

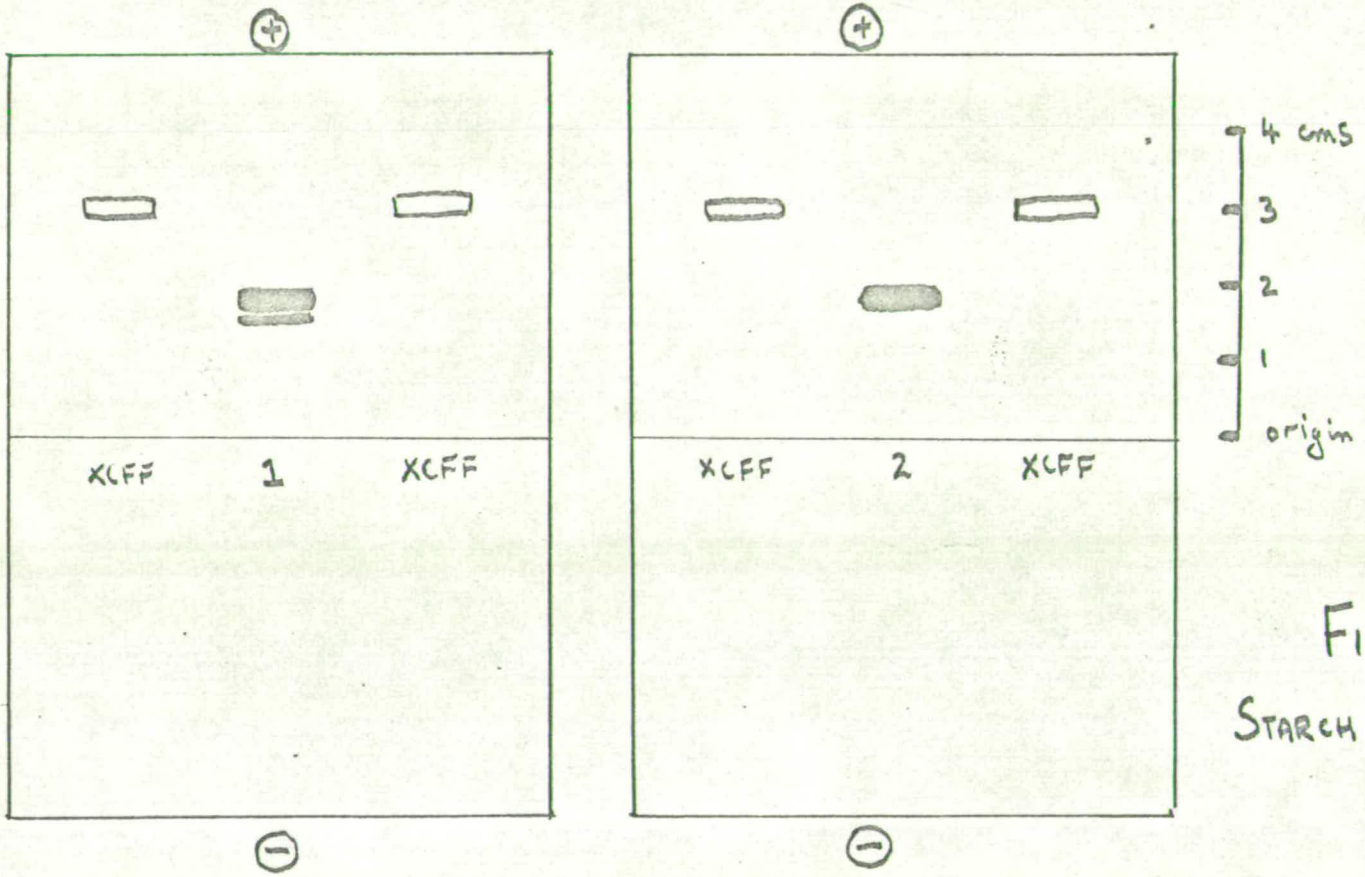


FIGURE 7

STARCH GEL ELECTROPHORESIS

1 β-lactamase before final chromatography

2 β-lactamase after final chromatography

Table 4. Summary of Six β-Lactamase Purifications

At each step the total activity is expressed in /(units) x 10^{-6} and the specific activity is expressed in (units/mg) x 10^{-4}. The final yield is expressed in mgs.

Starting material	Total activity	0.7	12.2	13.0[6]	119	78.3	114
	Specific "	1.1	1.5	0.9	0.9	0.6	1.3
After DEAE-cellulose chromatography		1.0[1]	11.5[1]	11.0	112	81.0	103
		2.5	2.2	1.4	2.6	1.4	2.5
After gel filtration		1.0	9.3	7.9	106	72.4	98.7
		13.0	15.5	9.4	15.9	14.1	15.9
After second chromatography		-	9.3	4.5	102[2]	67.7	91.3
			19.3	7.6	18.5	16.4	18.9
Yield of β-lactamase		6.5[3]	48	59	550	240[4]	483[5]

1 Initial chromatography in phosphate buffer pH 7.

2 Rechromatography; specific activity 18.3

3 Chromatographed at pH 6; specific activity 8.0.

4 Third DEAE-cellulose chromatography; 40% of preparation lost; specific activity 19.0.

5 Some activity lost by rotary evaporation.

6 This preparation was done at room temperature.

molarities were found to be critical for good results. On one occasion a third chromatographic step was necessary, but this could be avoided, with care (Table 4). Figure 7 shows the results of starch gel electrophoresis before and after the final purification step.

Several purifications are outlined in Table 4 and Tables 2 and 3 in Appendix I give more quantitative results of a single purification. Yields of E.coli β-lactamase of 60-65% and specific activities of 18.5 - 19.0 x 10^4 U per mg were routinely obtained.

In general, recoveries were better at 4°C than at room temeperature (Table 4). In the larger purifications, the Sephadex column was normally run at room temperature for convenience; otherwise, the purifications were carried out at 4°C. This may explain the losses in activity at the Sephadex stage.

In an early purification, β-lactamase was found to pass unretarded through a CM-cellulose column at pH 6.2. In addition, there was a loss of activity, due to prolonged exposure to this pH, which was partially reversed by dialysis against pH 7 buffer. Subsequently, the iso-electric point of β-lactamase was found, by starch gel electrophoresis at various pH values, to be at pH 5.4 with o.02 M gel buffers and at pH 5.8 with 0.05 M gel buffers.

The value of large-scale purification can be seen by comparing the later attempts with the first one in Table 4: a considerably higher specific activity was eventually obtained. This may be partly explained by the lack of material for really conclusive gel electrophoresis and amino acid analysis, from a small preparation. Over and above this, there does appear to be an improvement which is due to the larger amounts of material handled.

Criteria of Purity

E.coli β-lactamase was homogeneous as judged by starch gel electrophoresis at pH 8.5 and at pH 4.0. In my experience, about 0.04 mg of β-lactamase would give a faint but distinct 1 cm band in a starch gel slice stained for protein. In later purifications, 1 mg samples of protein were used for starch gel electrophoresis. Thus the absence of any contaminating protein bands suggests that any impurities are present at less than 4% of the preparation.

On SDS-polyacrylamide gels, a very faint band, corresponding to a very low molecular weight contaminant, could be seen.

The protein was homogeneous as judged by rechromatography on DEAE-cellulose (Table 4) and on Sephadex G-75 in both pH 7 buffer and 50% formic acid.

Enzymic Properties

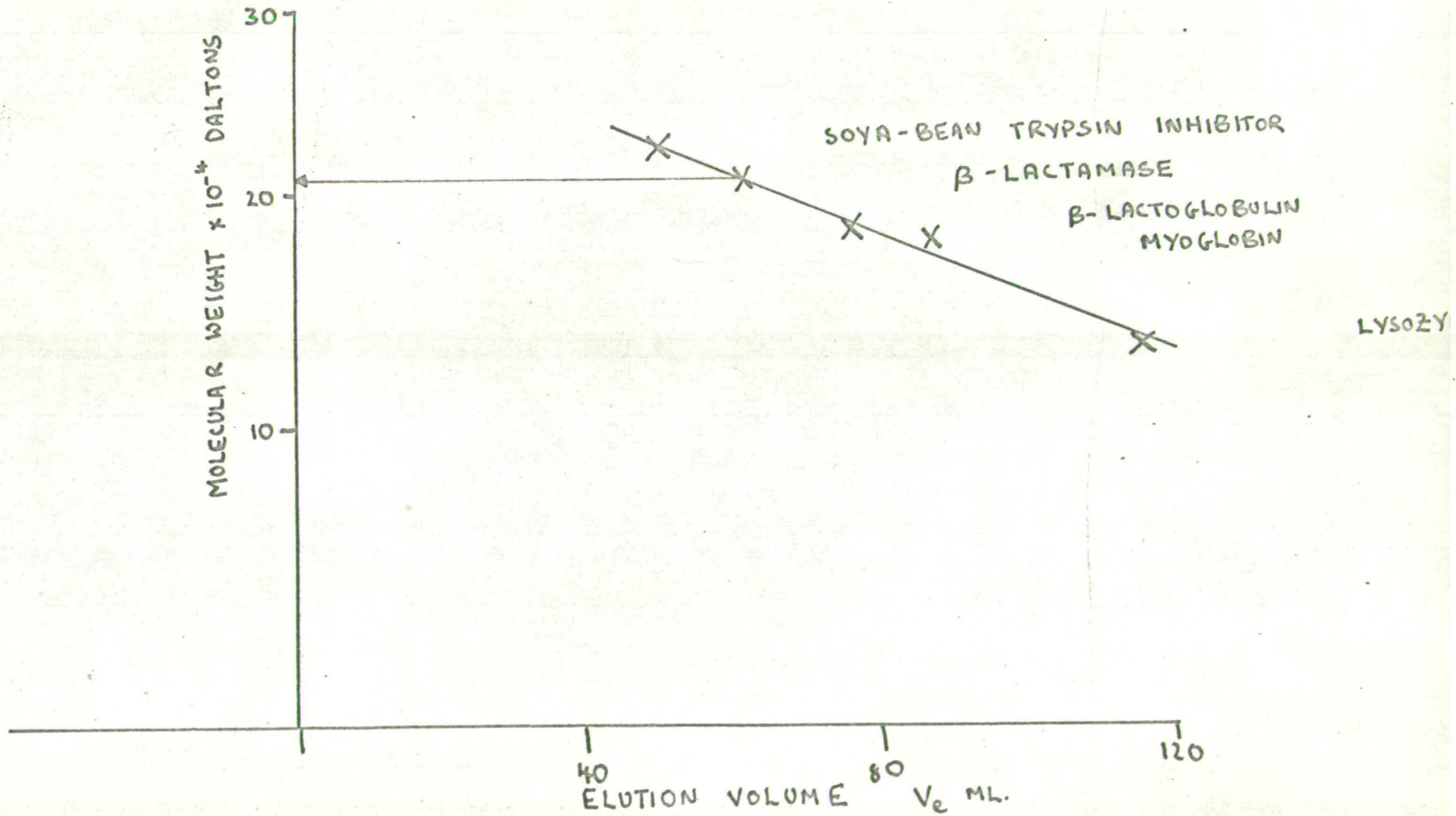
One sample of the purified enzyme had a specific activity of 18.5×10^4 units per mg of protein (3.1×10^3 International Units per mg).

The Michaelis parameters for this enzyme preparation were determined by measuring the initial rates of reaction at various substrate concentrations with the micro-assay, and using double reciprocal plots to calculate K_m and V (Lineweaver and Burk, 1934). For sodium benzylpenicillin at pH 6.90, K_m was 12 μmole and the molecular activity (V) was 6×10^4 moles per mole enzyme per minute.

The relative activities with respect to benzylpenicillin, ampicillin and ceporin were 100%, 162% and 125% respectively (J. Fleming, personal communication).

These relative activities are almost identical to the earlier,

FIGURE 8


30
20
10
MOLECULAR WEIGHT x 10^{-4} DALTONS
SOYA-BEAN TRYPSIN INHIBITOR
β-LACTAMASE
β-LACTOGLOBULIN
MYOGLOBIN
LYSOZY
40
80
120
ELUTION VOLUME V_e ML.

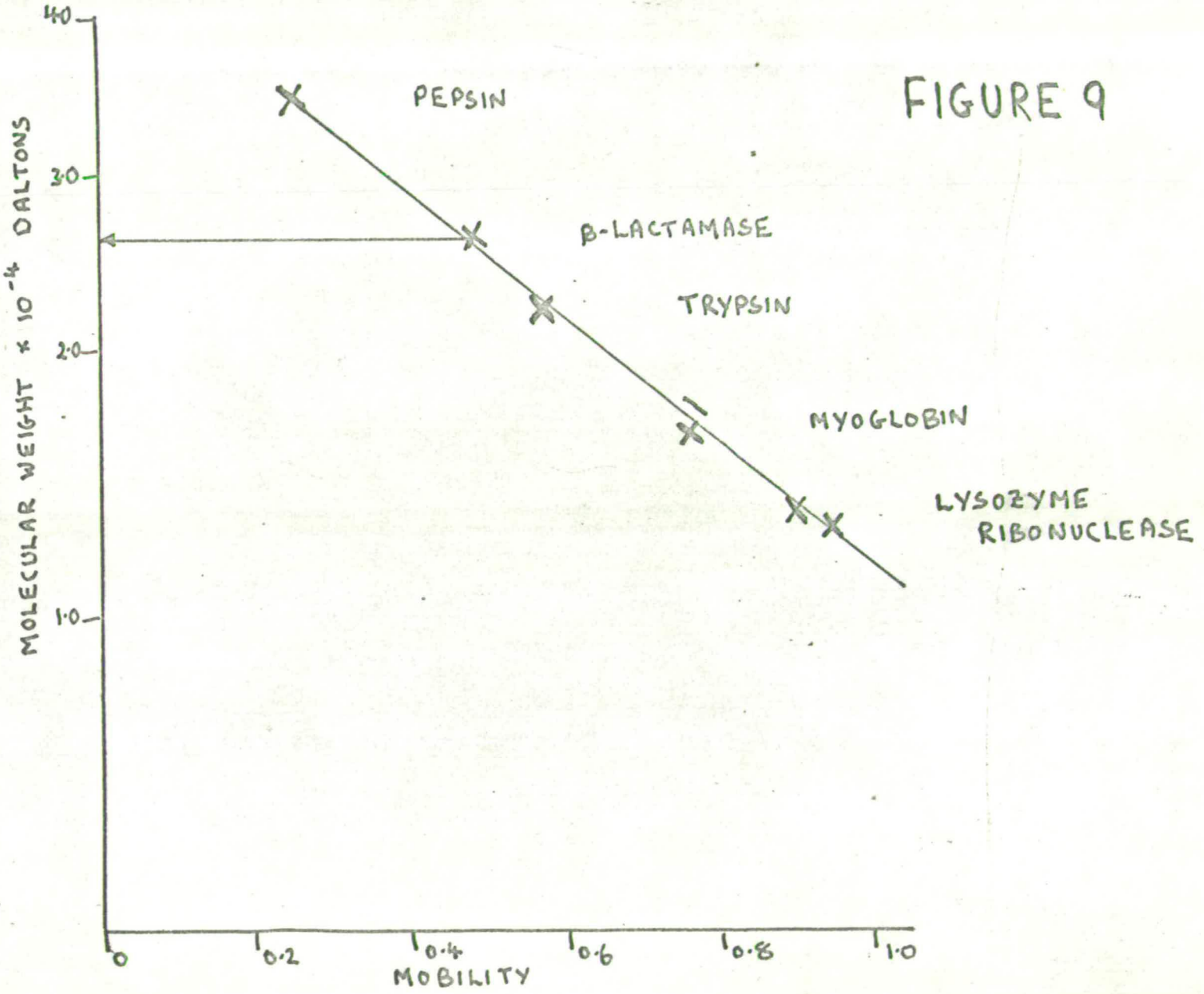


FIGURE 9
40
30
20
1·0
MOLECULAR WEIGHT × 10^{-4} DALTONS
PEPSIN
β-LACTAMASE
TRYPSIN
MYOGLOBIN
LYSOZYME
RIBONUCLEASE
0
0·2
0·4
0·6
0·8
1·0
MOBILITY

published values and K_m at pH 5.85, which is 29 μmole (Chapter X), is comparable with the earlier value of 25 μmole at pH 5.9 (Datta and Richmond, 1966).

Molecular Weight

Figures 8 and 9 are the semi-logarithmic plots of molecular weight against V_e and against mobility on SDS-gel electrophoresis respectively, for β-lactamase and various standard proteins. The respective values deduced for the molecular weight of E.coli β-lactamase are 2.1×10^4 and $2.7\ 10^4$ daltons.

From the amino-acid analyses (next section), a molecular weight of 2.0×10^4 daltons was deduced, as being in approximate agreement with the other determinations. The amino-acid composition, in residues per molecule of protein assuming this molecular weight, includes 9 lysine and 11 arginine residues. Thus 21 (9 + 11 + 1) tryptic peptides would be expected, which is approximately the number which have been isolated. (Chapter V). In addition, there are 6 methionine residues and 7 cyanogen bromide peptides (Chapter VIII).

Anomalous molecular weights can be deduced from the gel-filtration method (Andrews, 1964). Recently, it has been shown that charge alterations, due to maleylation, cause alterations in the mobility of proteins on SDS-polyacrylamide gel electrophoresis. (Tung and Knight, 1970). Thus the discrepancy in the results obtained by these methods is not very surprising. Their chief value is in indicating that the molecular weight calculated from the amino-acid composition is approximately correct.

The previously reported (Datta and Richmond, 1965) molecular weight of 1.7×10^4 daltons was determined with small quantities of a preparation which may well have contained less than 50%

β-lactamase and this may be considerably in error.

The mean value of the four determinations is 2.1×10^4 daltons.

This value for the molecular weight of E.coli TEM β-lactamase is comparable with the values for the enzymes isolated by Sawai et al. (1970), Yamagishi et al. (1969) and Lindqvist and Nordström (1970).

Amino-Acid Composition

Duplicate samples of approximately 1 mg were hydrolysed for 24, 48 and 96 hours. Amino-acid analysis was by the two-column method (Benson and Patterson, 1965). Results were calculated in terms of micromoles of amino-acid applied to each column and normalised using the internal standard. Examination of these results in conjunction with preliminary amino-acid sequence data and with the molecular weight determinations described above indicated that there were four residues of histidine, four residues of phenylalanine, seven residues of proline and nine residues of lysine per molecule of protein. Accordingly, for each analysis, the total amount in micromoles of each of these amino-acids was added and divided by 24 (4 + 4 + 7 + 9) to give the amount, in micromoles, of a single residue. This value was divided into the number of micromoles of each amino-acid to give the number of residues of that amino-acid per molecule of protein. The mean of each of these values is given in Table 5. The final column in Table 5 gives the number of residues of each amino-acid per molecule of protein, to the nearest whole number. These values are mostly the means of each of the corresponding values in the first three columns. The values for serine and threonine were deduced by extrapolation to zero time. There is no evidence for a slow release of valine or

Table 5. Amino-Acid Composition of *E.coli* β-lactamase

Residues in mols/mol β-lactamase

	24 h	48 h	96 h	Mean
lys	9.0	9.1	8.9	9
his	3.9	4.0	3.9	4
arg	10.7	10.3	10.4	11
asp	17.2	17.0	17.4	17
thr	13.2	12.5	11.5	14 *
ser	9.8	8.6	7.1	11 *
glu	19.5	19.1	18.8	19
pro	7.3	7.0	7.2	7
gly	14.6	14.0	14.6	14
ala	17.2	15.8	15.6	16
val	9.0	8.8	9.1	9
met	5.5	5.0	5.3	6 *
ile	8.9	8.8	9.1	9
leu	20.2	19.0	19.2	19
tyr	3.1	3.1	2.7	3
phe	4.0	4.0	4.0	4
cys				1 *
trp				3 *
			Total	176

* See text for details.

isoleucine. Some values for methionine are low but the evidence from cyanogen bromide hydrolysis of the protein (Chapter VIII) indicates that there are 6 residues per molecule. A low tyrosine recovery in one case may indicate incomplete evacuation of the sample tube prior to hydrolysis. There is some discrepancy between the various alanine and leucine values. This may be due to the fact that the 24 hr hydrolysates were of samples from a different batch of protein, but the deviations are within the limits of the reproducibility of the method. In the course of sequence studies, some evidence was found for the association of free amino-acids with the protein, which may also influence the validity of these results.

Carboxymethylated β-lactamase contained 0.9 mole of carboxymethyl cysteine per mole of protein. There was also some carboxymethylation of histidine. The analysis of a sample of β-lactamase oxidised with performic acid (Chapter IV) showed approximately 0.7 moles/mole of cysteic acid.

The chemical determination of tryptophan gave a value of 3.1 mole per mole of protein. The molar extinction coefficient of the protein was 21.4×10^3 at 280 nm; the molar extinction coefficient calculated on the basis of 3 moles tyrosine and 3 moles tryptophan per mole is 20.8×10^3 (Beavan and Holiday, 1952).

The reducing sugar content of the protein was found to be 0.8 and 0.4 mole/mole for two different samples.

Summation of residues and residue weights for E.coli β-lactamase gives 176 residues and a molecular weight of 20×10^4 daltons. The limitations discussed above and in Chapter IV preclude an accurate amino-acid composition based upon amino-acid

analysis; the complete amino-acid sequence is the only reliable source of this parameter.

CHAPTER III.

THE TERMINAL REGIONS OF *E.COLI* β-LACTAMASE

Methods

Fluorodinitrobenzene (Sanger, 1945) was used to investigate the N-terminus of β-lactamase. The procedures used were those described by Fraenkel-Conrat *et al.*, (1955).

0.5 μmol of protein was dissolved in 0.5 ml of 0.2 M-NEM-Acetate buffer, 8 M in urea in a screw-cap tube and 0.040 ml of FDNB (which is liquid when stored at 37°C) was added. The tube was flushed with nitrogen, sealed and shaken at 37°C overnight. This procedure normally caused some precipitation of the protein. The excess reagent was extracted with ether, dialysed against water, and freeze-dried and transferred to a test tube. The solid was dissolved in 0.3 ml concentrated HCl and 0.3 ml of water was added. It was sealed under vacuum and incubated at 105°C, normally for 24 h. It was then opened and extracted three times with 0.5 ml ether, which was back-extracted twice with 1 ml of 1M-HCl and dried in a stream of nitrogen. A portion of the residue, dissolved in ethanol, was chromatographed in two dimensions on a 25 cm square of Whatman No.1 paper, in parallel with standard Dnp-amino acids. The first dimension was 2-methylbutam-2-ol:2M-ammonia (4:1) (ascending) for 16 h and the second was 1.5M-sodium phosphate pH 6.0 (ascending) for 8 h (Ambler, 1963a; Levy, 1954).

In one experiment, the remainder of the residue was hydrolysed *in vacuo* at 105°C with 1M-NaOH (Snell and Offord, 1972) for 24 h. The hydrolysate was adjusted to pH 2.2 and 1.5 ml and was analysed for amino-acids.

Two variants on the Dns-Cl reaction were also used with β-lactamase. In the first (Gray, 1967), 0.1 μmole of protein was dissolved in 0.1 ml of 0.1 M sodium bicarbonate pH 8.5, 8 M in urea and 0.02 ml of saturated Dns-Cl in acetone was added. Incubation was in a sealed tube, on a shaker, for 8 h after which the solution was desalted, dried down and hydrolysed *in vacuo* at 105°C with 6N-HCl. The Dns-amino-acids were identified by electrophoresis at pH 4.38 (Chapter IV).

The other method (K. Weber, unpublished) involved reaction with Dns-Cl in 0.1 M-$NaHCO_3$ pH 9.3 containing 10% SDS, acid hydrolysis and then extraction of Dns-amino-acids with wet ethyl acetate. This reagent does not extract Dns-NH_2, which is often present in large amounts and which interferes with the separation and identification of the Dns-amino-acids. Nor does it extract the Dns-his, Dns-ε-lys or Dns-arg, so portions of the original hydrolysate were run in parallel with the extracts on pH 4.38 electrophoresis (Chapter IV).

Inconclusive results with these techniques prompted an analysis for possible N-acetyl- and N-formyl groups (Schmer and Kreil, 1969). 0.05 μmole of β-lactamase was dissolved in 0.5 ml 0.1 M-HCl and dried down, to drive off free acetate. The residue was heated with 0.3 ml anhydrous hydrazine in a sealed tube at 105°C for 16 h and the hydrazine was removed over NaOH *in vacuo*. The residue was dissolved in 0.3 ml 0.2 M-sodium citrate pH 3.25 and reacted with 0.3 ml of ethanol saturated with Dns-Cl at 37°C, with shaking, for 24 h. It was dried down, dissolved in 0.3 ml water and extracted three times with 1 ml ether. The residue was subjected to electrophoresis at pH 4.38 with standard Dns-amino-acids as markers (Chapter IV).

Leucine aminopeptidase was diluted ten-fold into 0.2 M-ammonium acetate pH 8.5, 0.002 M in $MgCl_2$. 0.025 ml of this solution were added to protein samples (0.05 μmole) dissolved in the same buffer (Potts, 1967). Incubation was for 1-3 h at 37°C and the reaction was stopped by the addition of 2 drops of glacial acetic acid. Free amino-acids were identified by pH 2.0 electrophoresis or with the amino-acid analyser (Chapter IV). β-lactamase and LAP controls were always included.

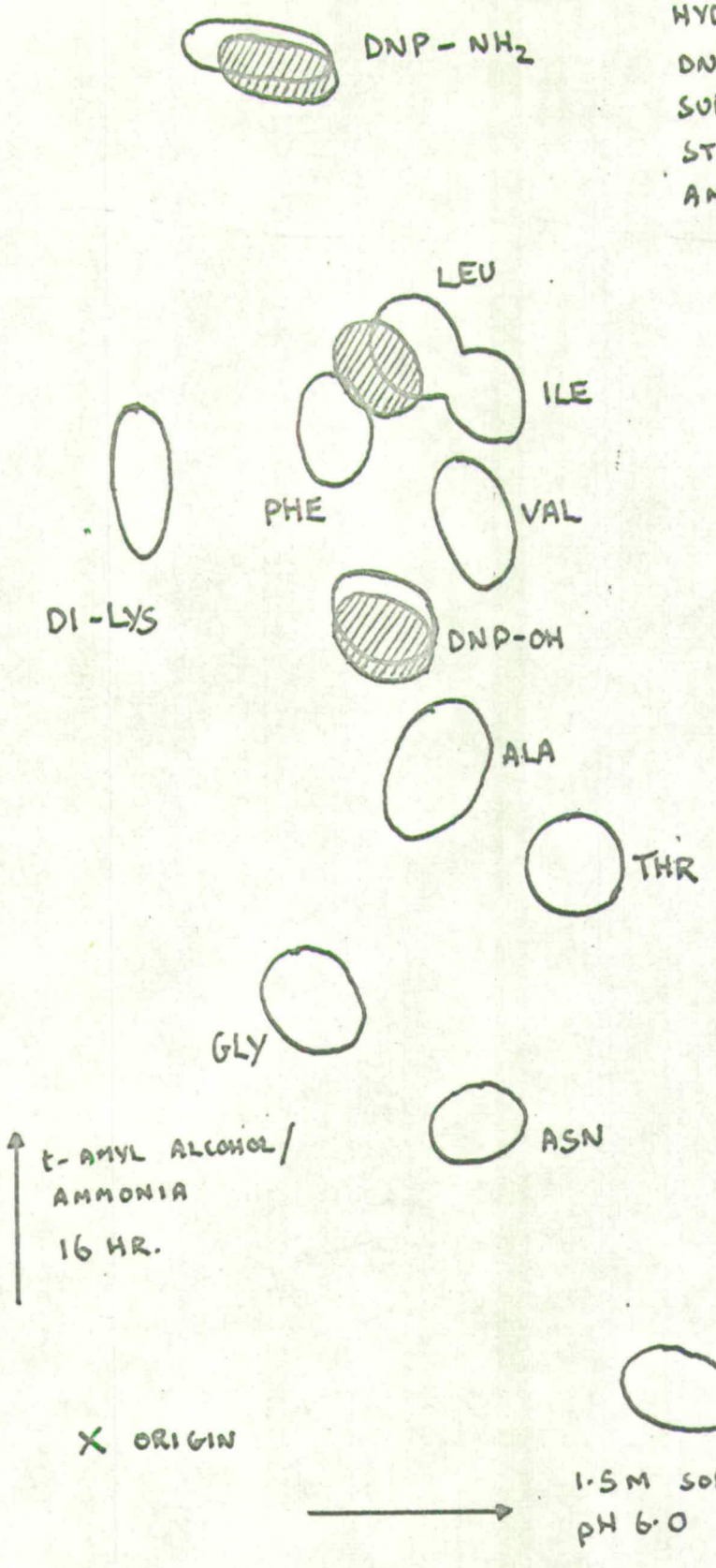
Carboxypeptidase A was prepared and used according to Ambler (1972). 0.050 ml of a suspension of CPA (60 mg/ml) was twice settled out of water to remove free amino-acids and then was suspended in 0.1 ml 1% $NaHCO_3$. If it did not dissolve, then 0.1 M-NaOH was added, drop by drop, with shaking and cooling, until it did so. An equivalent amount of 0.1 M-HCl was then added drop by drop and the solution was made up to 1.25 ml with 0.2 M-NEMAc pH 8.5. If the CPA precipitated during this procedure, it was discarded.

Protein samples of 0.05-0.15 μmole were dissolved in 0.25 ml of 0.2 M-NEMAc pH 8.5 and 0.005-0.1 ml of CPA was added. Incubation was from 0.5-4 h at 37°C and was stopped by adding 2 drops of glacial acetic acid. The amino-acids released were normally identified with the amino-acid analyser. The desiccated samples were redissolved in 0.2 M citrate buffer pH 2.2 and centrifuged to remove protein. β-lactamase and CPA controls were always included.

Carboxypeptidase B (Sigma, 11 mg/ml suspended in water) was diluted 20-fold into 0.2 M-NEMAc pH 8.5 (Ambler, 1967). To a 0.5 μmole sample of β-lactamase which had been extensively reacted with CPA, 0.050 ml of this solution was added and it was further

FIGURE 10

HYDROLYSATE OF DNP-β-LACTAMASE SUPERIMPOSED ON STANDARD DNP-AMINO ACIDS



incubated at 37°C.

The LAP, CPA and CPB used in these studies had all been pre-treated with DFP in an attempt to inhibit endoproteolytic activity.

Results

The first experiment with FDNB and native β-lactamase gave a weak spot which chromatographed in the same position as Dnp-leu and Dnp-ile standards.

A second experiment was performed with performic-acid-oxidised β-lactamase and β-lactoglobulin in parallel and Dnp-leu was tentatively identified in each case, although the β-lactamase spot was weak and could possibly have been Dnp-phe (Figure 10).

A third experiment employed native β-lactamase and ribonuclease in parallel. After the reaction with FDNB, one-half of the β-lactamase preparation was hydrolysed for 15 h and the other for 40 h. Chromatography of all three samples gave a strong, unidentified spot which was subsequently identified as ε-Dnp-lys by pH 3.5 electrophoresis. The ribonuclease control also gave a spot corresponding to bis-Dnp-lysine. Both hydrolysates of Dnp-β-lactamase gave spots corresponding to Dnp-leucine, although most of the 40-hr-hydrolysate was subjected to alkaline hydrolysis and amino-acid analysis. Leucine was the only significant amino-acid in the alkaline hydrolysate and the yield was approximately 40%, by comparison with the analysis of the aqueous phase left after the extraction of the Dnp-derivatives with ether.

The results obtained with the control samples of β-lactoglobulin and ribonuclease indicated that the method was technically satisfactory (Frank and Braunitzer, 1967; Smyth et al., 1963). With β-lactamase, Dnp-leucine was the only Dnp-amino-acid observed, apart

from -Dnp-lys in the third experiment, which was probably due to an imperfect extraction from the aqueous phase.

In all experiments with Dns-Cl, β-lactoglobulin was used as a control. Performing the reaction in urea was unsuccessful, but the SDS method gave Dns-leu from β-lactoglobulin. These experiments were carried out on native and oxidised samples.

Reaction of 0.025 μmol of β-lactamase with Dns-Cl using the technique described for determining the N-termini of peptides (Gray, 1967a, Chapter IV) gave rise to Dns-ser, identified by pH 4.38 and pH 2.0 electrophoresis and a weaker derivative corresponding to Dns-leu or -val on pH 4.38 electrophoresis, but not further identified.

Leucine amino peptidase did not release amino-acids from native β-lactamase when the reaction was carried out in 0.2 M-ammonium acetate 0.002 M in $MgCl_2$ nor when this buffer was made 2 M in urea (Hill and Smith, 1957).

A subsequent experiment with β-lactoglobulin and β-lactamase in parallel, with negative results in both cases, showed that the LAP used in these experiments was probably inactive.

In one of these experiments, the β-lactamase control gave the following results on amino-acid analysis: serine, 1.4 mole/mole; glycine, 0.9 mole/mole; alanine, 0.4 mole/mole.

The equivocal results with these experiments led to a search for a possible blocked N-terminus.

Schmer and Kreil (1969) claim that 1-acetyl-2-dansyl hydrazine migrates to the cathode on pH 4.38 electrophoresis more rapidly than any by-products of the hydrazinolysis and dansylation of a protein, and that 1-formyl-2-dansyl hydrozine migrates with $Dns-NH_2$. No

Table 6.

β-lactamase µmol	CPA ml.	Time h	ala	leu	gly	ser	ile	try	lys	his
0.15	0.005	2	-	-	-	-	-	-	0.4	1.0
0.08	0.02	0.5	-	-	-	0.3	0.6	1.0	1.0	1.0
0.15	0.060	4	0.5	0.5	0.6	0.7	1.0	0.9	1.0	1.0

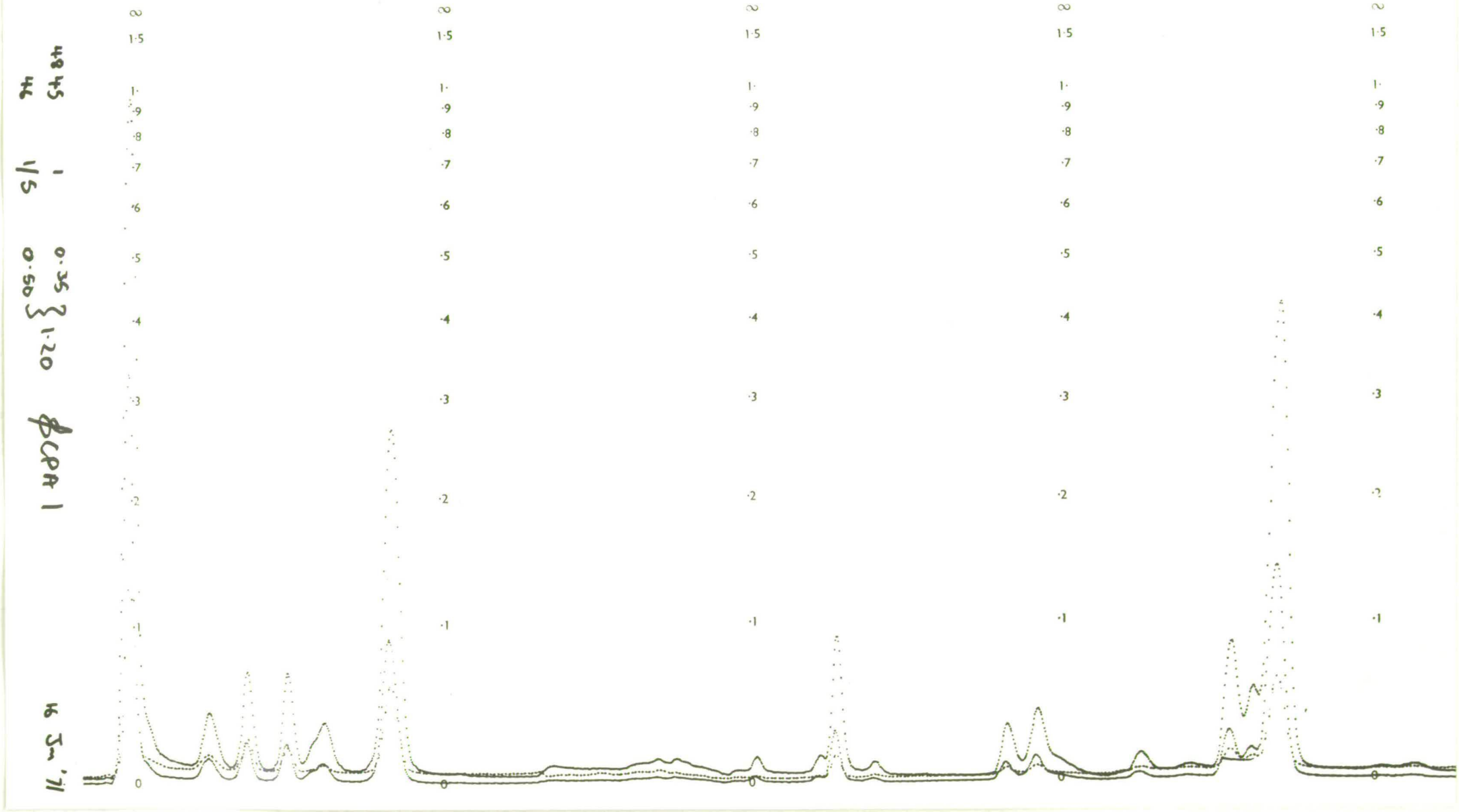


Figure 11 CPA experiment

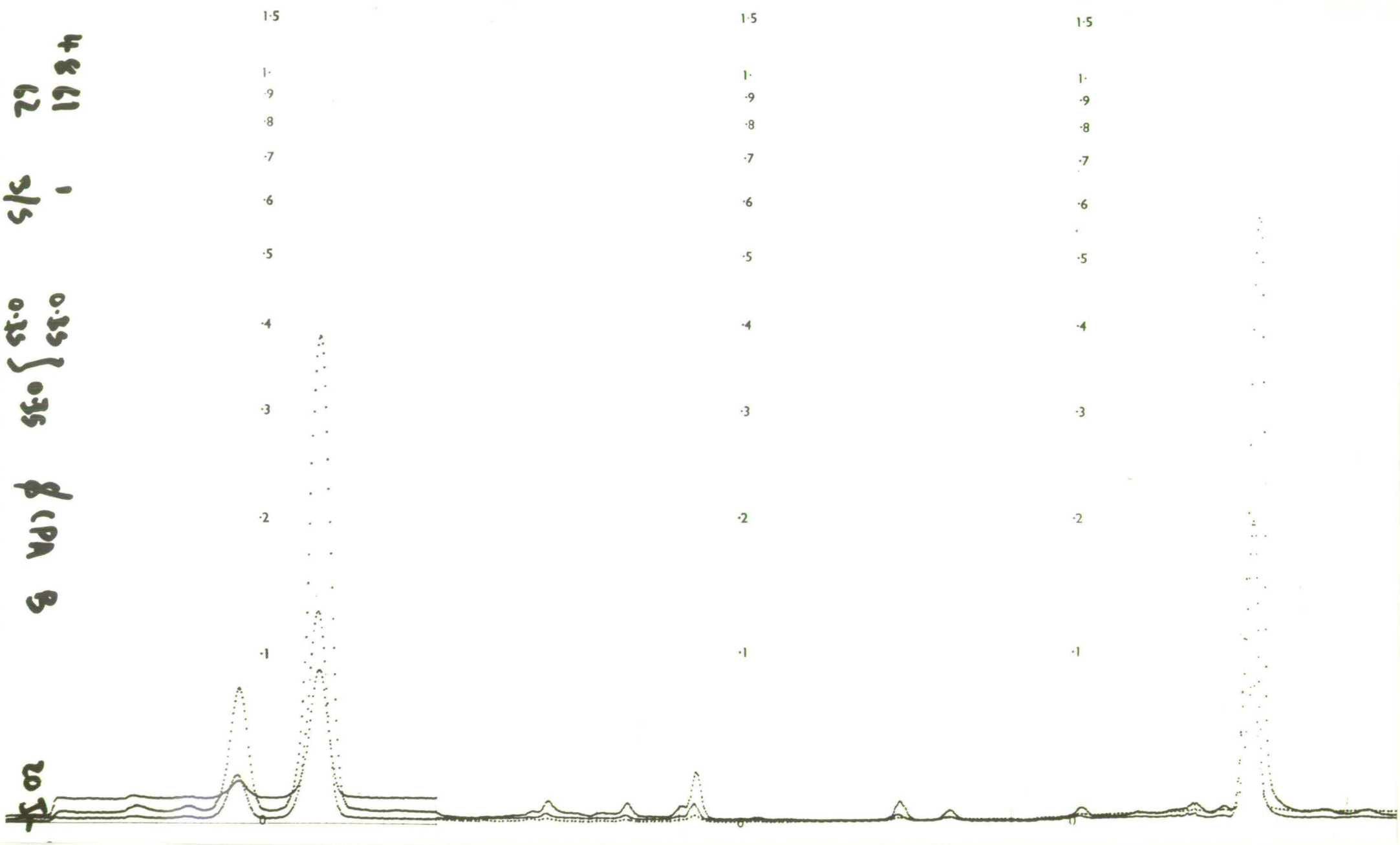


Figure 11 CPA blank

spot corresponding to the acetyl-derivative was observed, but there was some Dns-NH_2 which would perhaps obscure a possible formyl-derivative. Incubation of β-lactamase in 1 M-HCl in methanol for 1.5 h at room temperature failed to generate new NH_2-termini. This technique has been used to remove N-formyl residues (Yang, 1958; quoted in Needleman, 1970, p.89).

Ikenaka et al. (1966) have converted the N-terminal pyroglutamic acid of orosomucoid to glutamic acid by hydrolysis with 1 M-NaOH for 72 h at 25°C, but some peptide bond hydrolysis was also detected. A similar 48 h treatment of β-lactamase followed by Dns-Cl treatment failed to reveal glutamic acid or any other amino-acid as N-terminus.

The presence of carbohydrate material in β-lactamase (Chapter II) suggested that this might be a conceivable, if somewhat unusual, derivative of the N-terminal amino-acid. The variability of the carbohydrate content from one sample to another renders this hypothesis unlikely.

The results of experiments with CPA are shown in Table 6. Each of the three sets of results were obtained with a different preparation of CPA. The amino-acid analyses were normalised to 1.0 residue of histidine for each amino-acid after subtracting the total amounts of that amino acid in the control experiments. Serine was always very high in the β-lactamase control; in one case there was 0.5 mole serine/mole protein. There was almost no autolysis of CPA under these conditions. Figure II shows the amino-acid analyser charts from one of these experiments.

These results suggest that the C-terminal sequence of β-lactamase is -(ala, leu, gly)-ser-ile-try-lys-his.

Very extensive hydrolysis with CPA and CPB gave a very complex mixture of all the amino-acids and endoproteolytic activity in the CPB preparation was suspected.

The experiments with FDNB suggest that the N-terminal amino-acid of E.coli β-lactamase is leucine. The results of experiments with Dns-Cl can be explained by the relatively high levels of free amino-acid associated with β-lactamase and observed in other experiments. The Dns-Cl experiments were largely unsuccessful in dansylating the N-terminus of the protein but free and soluble serine was dansylated easily. Desalting or dialysis of Dns- or Dnp-β-lactamase would remove Dns-serine in most experiments.

There is no evidence for any other amino-acid as N-terminus, or for a "blocked" N-terminus.

The postulated C-terminal sequence of E.coli β-lactamase is:

(ala, leu, gly)-ser-ile-try-lys-his.

These experiments also serve as additional criteria of purity for E.coli β-lactamase.

CHAPTER IV.

TECHNIQUES IN AMINO-ACID SEQUENCE DETERMINATION

A. Generation of Peptides

The recognition of the specificity of various proteolytic enzymes and of the necessity for denaturation of proteins prior to enzymic hydrolysis (reviewed by Bergmann and Fruton, 1941) was utilised at an early stage in the sequencing of proteins (Sanger, 1952). In some cases at least proteolytic enzymes will act upon native proteins and seem to cause denaturation, perhaps simply by the cleavage of a few peptide bonds (Linderstrøm-Lang, 1951).

Various denaturation techniques have been developed (Bennett, 1967) and many have been employed upon E.coli β-lactamase. Heat denaturation of small samples produced precipitates which were not solubilised by trypsin but dissolving in 1 M-acetic acid and adjusting to pH 8.5 was more successful. Trial digestion and one-dimensional electrophoresis of these samples and of native β-lactamase showed results which were qualitatively and quantitatively similar. Digestion times of 2 to 8 hours also gave similar results.

Oxidative denaturation with performic acid (Hirs, 1956) is often very effective, presumably because methionine residues, which are often in the interior of the three-dimensional structure of a protein, are converted into hydrophilic methionine sulphone residues. Cysteine is oxidised to cysteic acid, which is convenient for sequence studies, but tryptophan is destroyed.

Performic acid was prepared by adding one part of 100-volume hydrogen peroxide to 19 parts of 98% formic acid and standing at 4°C for 2 h. To protein samples (20 mg/ml in 98% formic acid)

was added an equal volume of performic acid. After incubating at room temperature for 2 h, the samples were desiccated or diluted and freeze-dried. Traces of tyrosine monochloride ("tyr-X") were observed on analysis of oxidised samples (Sanger and Thompson, 1963).

Small samples of oxidised β-lactamase were digested more fully by trypsin than was native protein. On the preparative scale, the oxidised protein was often largely insoluble at pH 8.5 and so it was dissolved in 0.3 M ammonium carbonate pH 8.3, 6M in urea, diluted 3-fold and incubated with trypsin added in small aliquots at intervals (Helinski and Yanofsky, 1962).

Pre-incubation for 1 h in 8 M urea followed by de-salting on Sephadex G-25 in 0.1 M-ammonia with the trypsin already in the receiving vessel and periodic adjustment of the eluate to pH 8.5 has also been employed in the digestion of β-lactamase.

Similar methods of denaturation have been employed prior to chymotryptic digestion. Pre-incubation at pH 2.0 was carried out prior to peptic digestion. These conditions are summarised in Table 8.

Trypsin (Worthington SR 618.8) was treated with DPCC to inhibit chymotryptic activity prior to use (Erlanger and Cohen, 1963). 40 mg of trypsin was dissolved in 4.0 ml 0.1 M-tris-hydrochloride buffer pH 8.0, 0.1 M in $CaCl_2$. 10 mg of DPCC was dissolved in 1.7 ml acetone and 0.02 ml was added to the trypsin solution, which was then incubated for 90 min at room temperature, centrifuged and decanted. Routinely, 4.0 ml of this solution was freshly made for a "major" tryptic digest and the remainder was stored frozen for use in the digestion of peptides. The ratio of protein to trypsin in these experiments was normally 40 to 1 and the protein was

Table 8. Summary of β-Lactamase Digests

μmol PROTEIN	DENATURATION	DIGESTION CONDITIONS (37°C)	INITIAL FRACTIONATION
2	Performic acid	1mg DPCC-trypsin, 4h	G-25 in 5% formic acid
8	None	4mg " " "	Cation-exchange chromatography
5	Performic acid	3mg " " " , in urea	" " "
5	" "	5x0.5mg " " 2h, in urea*	G-25 in 5% formic acid
8	8 M-urea	4mg " " 3h	Cation-exchange chromatography
8	ETPA	4mg " " 4h	G-25 in 0.1 M-ammonia
2	None	1mg SBTI-chymotrypsin, 4h	G-25 in 5% formic acid
7.5	Performic acid	4mg " " "	" " " " "
11	8 M-urea	5mg " " 16h	Cation-exchange chromatography
9.5	Acid	5mg pepsin, 4h	G-25 in 0.1 M-ammonia
5		160mg CNBr, 24h, 30°C	G-25 in 5% formic acid
2.5		100mg " 48h, 30°C	G-50 in 50% formic acid
ca.20		500mg " 36h, 20°C	G-50 in 1 M-sodium propionate
5		150mg " 30h, 20°C	G-50 in 50% formic acid
5		150mg " " "	" " " " "

* 5 portions added at 15' intervals.

dissolved at pH 8.5, in 0.2 M ammonium acetate and at 5-10 mg/ml.

Despite the DPCC treatment, "chymotrypsin-like" cleavages have been observed in tryptic digests. An elegant and convincing explanation of this phenomenon has recently been brought forward (Keil-Dlouhá et al., 1971). The autolytic cleavage of a particularly labile peptide bond converts β-trypsin to α-trypsin, which is further autolysed to form Ψ-trypsin, or pseudotrypsin, which is partially active as an esterase and has an altered, broader specificity with polypeptides, cleaving bonds at the C-terminus of basic and hydrophobic amino-acids.

In the course of attempts to remove pseudotrypsin from trypsin by ion-exchange chromatography (Maroux et al., 1962), it was discovered that a preparation which displays both tryptic and pseudotryptic activity at pH 8.5 displays only tryptic activity at pH 4.0.

The following assay was suggested by Mr. G. Pettigrew. A 10 mg/ml suspension of glucagon (Sigma) in water was used as substrate. 0.010 ml of this suspension was reacted with 0.010 ml of trypsin solution (1 mg/ml) in 0.10 ml buffer. After 24 h at 37°C the tubes were dried in vacuo and reacted with Dns-Cl (Gray, 1972a). The Dns-amino-acids were separated by electrophoresis at pH 4.38 and those present were estimated semi-quantitatively (Table 7).

Table 7.

pH	9.7	8.3	7.0	5.2	4.7	4.0	GB
His	++++	++++	++++	++++	++++	++++	++++
Ala	++++	++++	+++	+++	++	++	-
Thr	++	++	+	-	-	-	-
α-Tyr	+	++	++	++	+	++	++
bisTyr	+	+	+	+	+	+	-
Arg	++++	++++	++++	++++	++++	++++	-
Leu	+++	+++	++	+	-	-	-

GB Glucagon Blank.

α-Tyr was very weak.

Histidine is the N-terminal residue of glucagon. Only tyrosine, arginine and alanine N-termini should be generated as the result of cleavage of glucagon by α- or β-trypsin (Keil-Dlouhá et al., 1971). Ψ -trypsin will, however, produce threonine and leucine N-termini in addition to these. Thus the absence of Dns-leu and Dns-thr at low pH may indicate a low pseudotryptic activity relative to tryptic activity. Trial digests of β-lactamase and of various cytochromes (G. Pettigrew and R.P. Ambler, personal communication) at pH 4.0 indicated that only limited digestion took place, but the technique may be useful in avoiding pseudotryptic activity in the sub-digestion of peptides.

40 mg of chymotrypsin (Worthington CD 16108) was dissolved in 3.0 ml of 0.2 M ammonium acetate pH 8.5 and 4 mg of SBTI in 1.0 ml of this buffer was added. After 1 h at 37°C, the preparation was

centrifuged and decanted (Ambler, 1963b). The use and storage of this enzyme was as for DPCC-trypsin.

Pepsin (Worthington) was simply dissolved in pH 2.0 buffer (2% formic acid in 8% acetic acid) and immediately added to the protein sample, in the same buffer at 10 mg/ml.

Incubation times for enzymic digestion varied from 2 h to 20 h (see Table 8). Dilution and shell-freezing prior to freeze-drying were normally used to stop the reaction.

Cyanogen bromide was also used to cleave the β-lactamase molecule (Gross and Witkop, 1962). In acid, CNBr reacts with methionine residues in proteins to form cyanosulphonium bromide derivatives which cyclise to form internal iminolactones of homoserine which decompose and cleave the peptide chain. The fragments have homoserine (in equilibrium with its lactone) at the C-terminus.

Generally, the β-lactamase was dissolved at 40 mg/ml in 50% (V/V) formic acid and one-and-a-half-times as much CNBr (by weight) in the same volume of 50% formic acid was added. This represents an approximate 50-fold excess of CNBr. After overnight reaction, less than 5% methionine remained.

In one experiment, an old sample of CNBr was used. After the reaction, the solution was purple in colour and there was considerable precipitation. About 15% methionine remained. It was difficult to solubilise the freeze-dried preparation and only the small CNBr-peptides were successfully purified.

Analysis of older samples of peptides and protein sometimes indicated some methionine sulphone. Thus it was felt that reduction of a protein sample prior to adding CNBr might improve the yields of peptide. An 8 M urea solution was deionised with

"Biodeminrolit" resin (Permutit), made 0.2 M with respect to Tris-hydrochloride and adjusted to pH 8.0 with 1 M-HCl. After bubbling nitrogen through this buffer, 25 ml was added to 580 mg β-lactamase in a screw-cap flask, which was flushed with nitrogen. The flask was placed on a balance and 100 mg of 2-mercaptoethanol was added with a Pasteur pipette, followed by a nitrogen flush. After 3 h at 37°, the protein was precipitated with 10% TCA, collected by centrifugation and washed with ether (Crestfield et al., 1963). Subsequently, the protein was not entirely soluble in formic acid. After the CNBr reaction, an attempt was made to fractionate the products on Sephadex G-50 in 1 M-sodium propionate.

B. Purification of Peptides

In some digests, Sephadex gel filtration was used as a preliminary purification step. Columns (100 cm x 1 cm) of Sephadex G-25 and G-50 (Pharmacia; fine or superfine grade) were packed as described in Chapter II. The chosen eluents were normally 5% (V/V) formic acid or 0.1 M-ammonia. 5% formic acid, 8 M in urea, was used to fractionate cyanogen bromide peptides but the removal of urea was a disadvantage; 50% formic acid was found to be most suitable, although care must be taken that proprietary synthetic column fittings are not over-exposed to this solvent. An attempt to fractionate cyanogen bromide peptides using Sephadex G-50 in 1,0 M-sodium propionate (G. Frank, personal communication) was unsatisfactory as there was some precipitation in the column.

Column effluents were continuously monitored with respect to optical absorbance at 254 nm (LKB "Uvicord") and small (1-2 ml)

fractions were collected. Portions of each fraction (0.04-0.08 ml) were subjected to paper electrophoresis at pH 6.5 to form a "peptide map", which was used to divide the fractions into a few pools which were fractionated by electrophoresis.

In some cases, ion-exchange chromatography was used as the initial fractionation procedure. The methods used were basically those of Schroeder (1967) and the equipment was largely from a defunct peptide analyser (Technicon).

The buffers used were:

(i) pH 3.1 pyridinium acetate, 0.2 M in pyridine.

(ii) pH 5.0 pyridinium acetate, 2 M in pyridine.

(iii) pH 5.6 pyridinium acetate, 8 M in pyridine.

The pyridine was re-distilled from ninhydrin.

The resin (Technicon "Chromobeads" Type P; 4% cross linked cation exchange resin) was washed with several volumes of water, with 0.2 M-NaOH, again with water, with 0.2 M-HCl, yet again with water and finally with starting buffer before each experiment. A column (30 cm x 0.9 cm) was packed with a positive-displacement pump and jacketed at 55^{o}C. A spare column mounted on an amino-acid analyser was most effective. The sample was dissolved in 1-2 ml of starting buffer (pH 3.1), allowed to flow on to the column under gravity, and washed in with more buffer. The column was developed initially with a linear gradient from pH 3.1 to pH 5.0, using the pump. A final wash with 25 ml of pH 5.6 buffer was used to remove any tightly bound peptides. The effluent was monitored continuously at 254 nm and fractions of 1-2 ml were collected. A peptide map was prepared as above.

Several trials were made using β-lactoglobulin (Pentex) digested

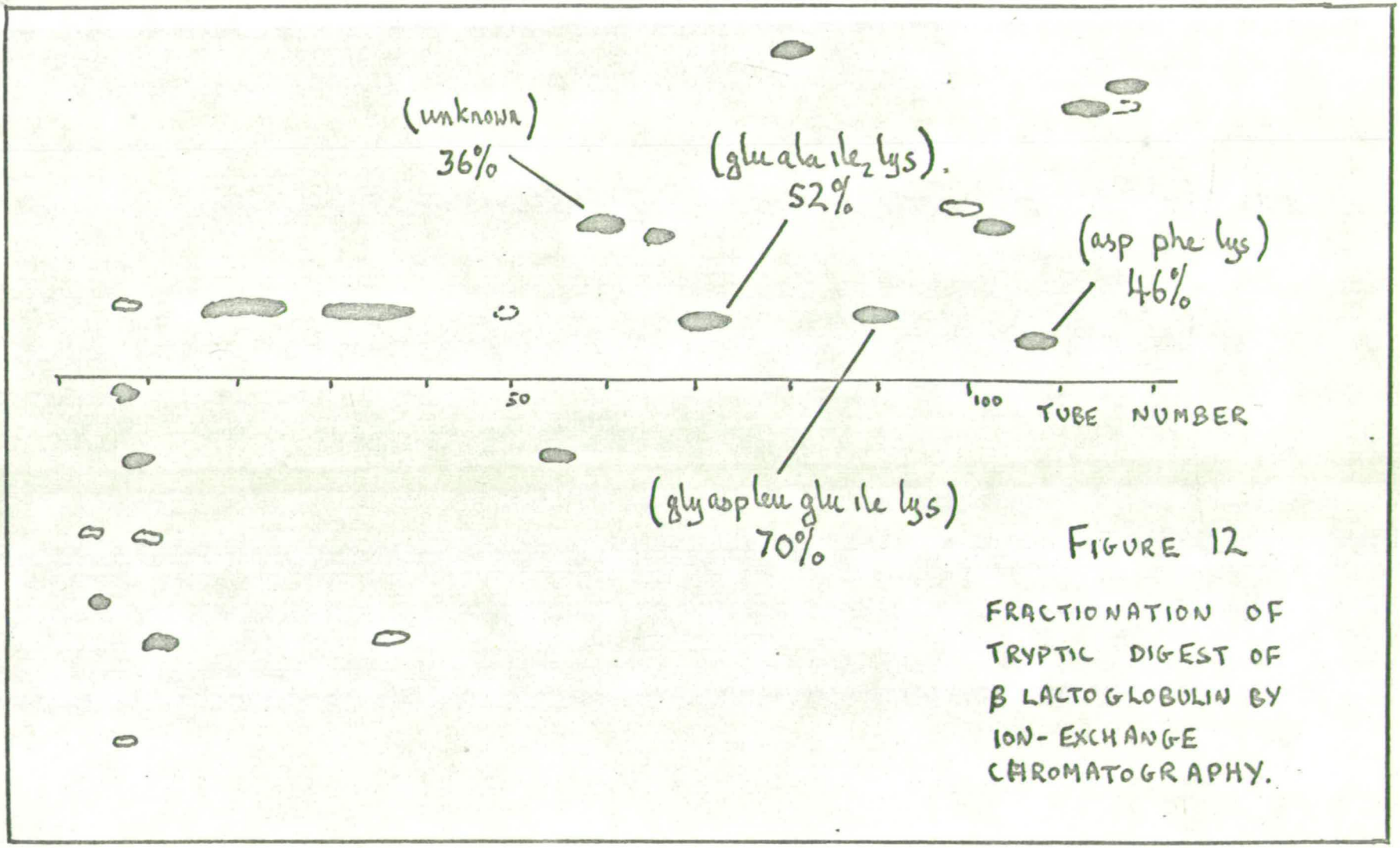


FIGURE 12

FRACTIONATION OF TRYPTIC DIGEST OF β LACTOGLOBULIN BY ION-EXCHANGE CHROMATOGRAPHY.

with trypsin. Figure 12 shows the peptide map of one such trial together with the results of analysis of some of the peptides fractionated; the peptides examined were not as pure as the peptide map seemed to indicate and were mostly small. Nevertheless, the yields were encouraging. In another trial, an attempt was made to improve the separation of the more acidic peptides by using 0.2 M pyridinium formate buffer pH 1.85 and 2 M buffer pH 4.95 to construct the gradient but there seemed to be a lower recovery of neutral and basic peptides. Instead, a shallow concave gradient of the original acetate buffers was used, the mixing vessel holding 150 ml of pH 3.1 buffer and the reservoir holding 100 ml of pH 5.0 buffer.

Figure 19 (Chapter V) shows the peptide map of the ion-exchange chromatography of a tryptic digest of β-lactamase. This was fairly reproducible.

There was always some insoluble material in digests prior to either initial purification step, It was removed by centrifugation and generally proved to be insoluble in 50% formic acid, in equal volumes of 5% formic acid and ethanol and in 8 M urea. In the case of one tryptic digest in which pre-incubation in urea and de-salting were used to denature the protein, the insoluble material was precipitated at the end of the digest with 10% TCA. The precipitate was dissolved in 1 ml of equal volumes of 5% formic acid and ethanol (R.P.Ambler, unpublished). It was applied to a column (50 cm x 0.5 cm) of Poragel A-25 (Waters Associates Ltd.) in this solvent. Poragel is stable and inert cross-linked styrene in bead form and is suitable for gel filtration in organic solvents as it does not shrink.

Invariably, further purification of peptides was necessary and

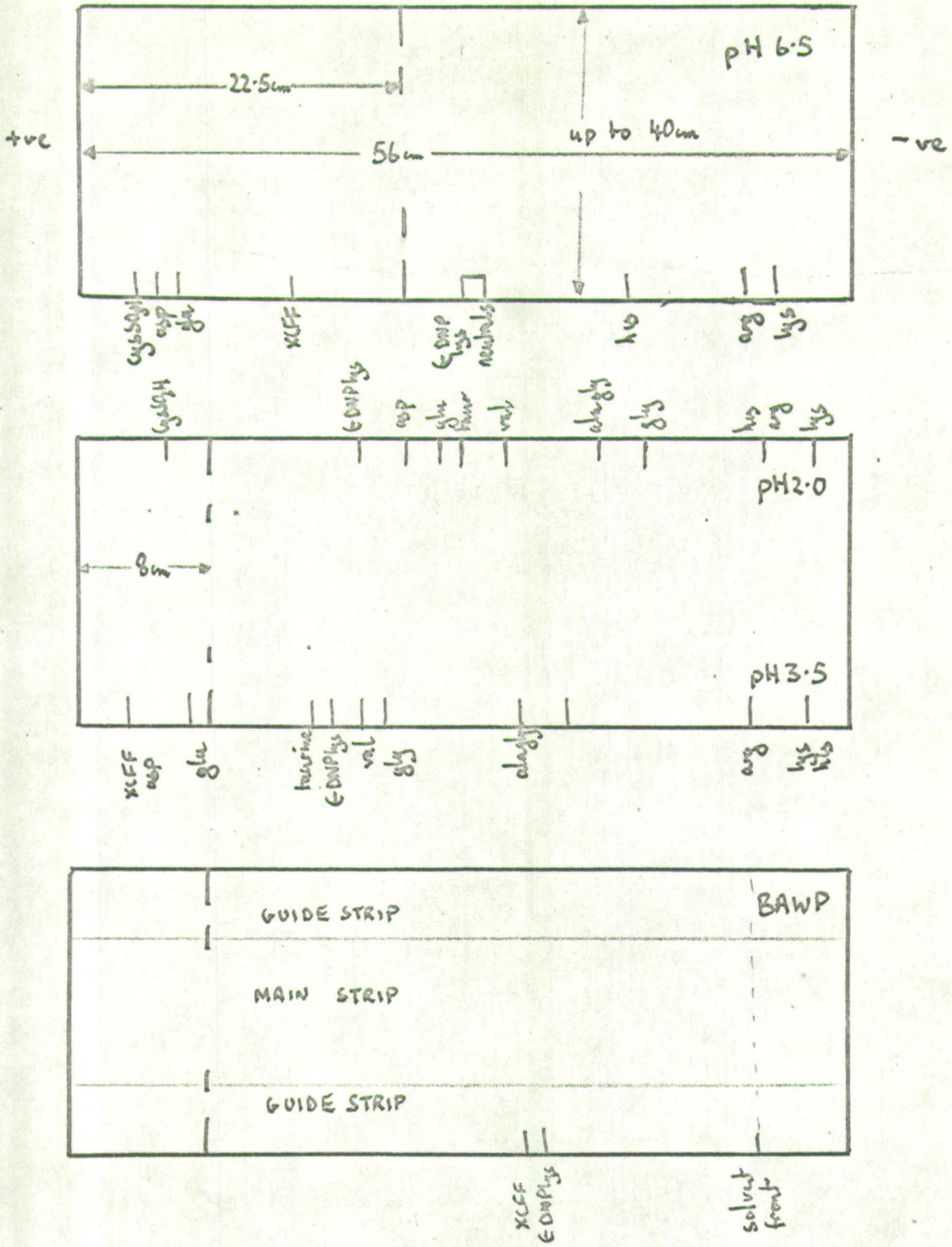


FIGURE 13 ELECTROPHORESIS AND CHROMATOGRAPHY

paper electrophoresis and chromatography were used. Electrophoresis was carried out with sheets of paper wetted with buffer and dipped in buffer troughs at either end and entirely immersed in an organic coolant (Michl, 1951). Electrophoresis was routinely at pH 6.5 (pyridine:acetic acid: water - 25:1:225; 8% pyridine in toluene as coolant; Ryle et al., 1955), at pH 3.5 (pyridine:acetic acid:water - 1:10:89; white spirit as coolant; Ryle et al., 1955), and at pH 2.0 (2% formic acid in 8% acetic acid; white spirit as coolant; Ambler, 1963b). Descending paper chromatography of peptides was carried out for 16 h using BAWP (Waley and Watson, 1953). A standard mixture of amino-acids and coloured markers was applied to each paper purification (Milstein and Milstein, 1968). The dimensions and layout of the various papers, and the eventual positions of the standards, are shown in Figure 13. Trial runs and weak samples were run on Whatman No.1 chromatography paper, and stronger preparative samples were run on Whatman 3 mm. The latter was preferred for peptide maps, as it is stronger. The loading of samples on papers was loosely based on a maximal loading of 0.05 μmole of whole, digested protein per cm for 3 mm paper, and about one quarter of this for No.1 paper. A potential difference of 2-3 kilo volts gave currents of up to 250 ma.

Peptides were located by the following methods:

Ultraviolet Fluorescence. Tryptophan and tyrosine peptides fluoresce if viewed under an ultraviolet light but other peptides can also fluoresce if warmed.

Ninhydrin. The paper was dipped through 0.2% fresh Ninhydrin (Merck) in acetone, dried in air and heated to 105°C for a few minutes (Toennies and Kolb, 1951). Material containing

amino-groups gave a dark blue colour. Glycine, and sometimes threonine and serine, N-termini gave a greenish-brown colour, if there was no other amino-group in the molecule. Similarly hydrophobic N-termini sometimes gave a weaker colour than normal. Peptides with no amino-groups did not give any colour at all. Poor colour yields were sometimes found with pH 3.5 electrophoretograms if the ninhydrin solution was not fresh.

Chlorination (Rydon and Smith, 1952). The paper was dipped in acetone and left in a chlorine atmosphere (generated from equal volumes of saturated, aqueous $KMnO_4$ and 2.4 M-HCl) until the ninhydrin colours disappeared. It was then hung in air for 10 min and sprayed with a solution made by boiling 5% starch in 0.025 M-KI and cooling. Material containing peptide bonds gave a very dark blue colour against a pale blue background. This technique was used routinely in the early stages of many purifications.

Tyrosine (Jepson and Smith, 1953). The paper was dipped through 0.1% 1-nitroso-2-naphthol in acetone, dried in air and then dipped through 10% nitric acid in acetone. When dry, it was heated in an oven with an extractor. Tyrosine-containing peptides gave a red colour. This technique could be used after ninhydrin but the paper could not be subsequently stained again.

Histidine (The Pauli reaction; Dent, 1947). Both sides of the paper were sprayed with a solution made by cooling and mixing equal volumes of 1% sulphanilic acid and 5% sodium nitrite. When dry, it was sprayed with 15% sodium carbonate solution, whereupon an orange colour was given by histidine-containing peptides and a darker red colour by tyrosine-containing peptides. This stain could be used after ninhydrin.

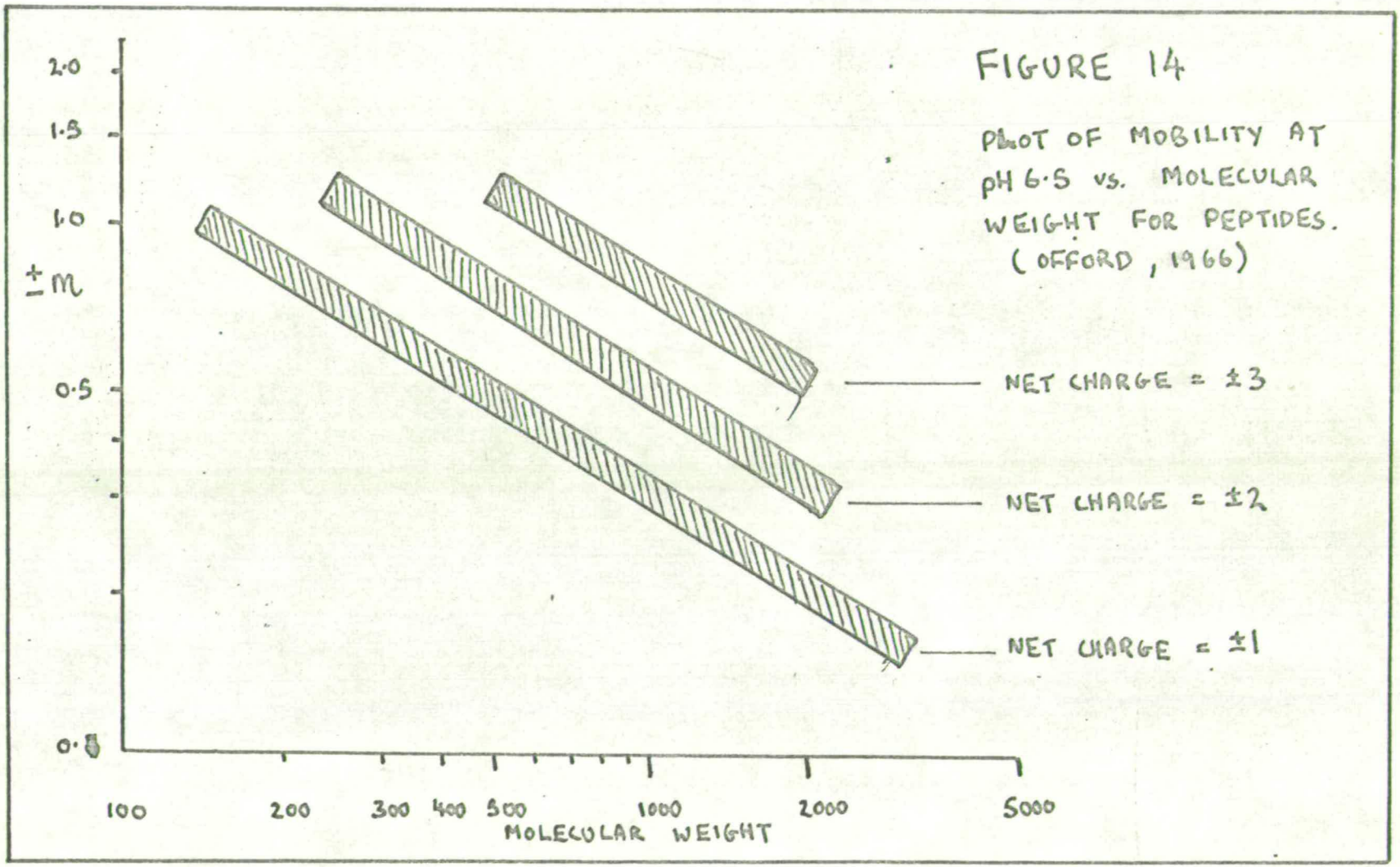


FIGURE 14

PLOT OF MOBILITY AT pH 6·5 vs. MOLECULAR WEIGHT FOR PEPTIDES. (OFFORD, 1966)

Tryptophan (Ehrlich's reagent; Dalgleish, 1952). The paper was dipped through a solution consisting of 9 parts of 4-dimethylamino-benzaldehyde in acetone and one part of concentrated HCl. It was dried in air and tryptophan-containing peptides gave a greyish-purple colour after about 5-10 min. This colour faded quickly. This stain could be used after ninhydrin.

Isatin (Acher, Fromageot and Jutisz, 1950). The paper was dipped through 0.2% isatin in acetone with 4% glacial acetic acid. On drying and heating, peptides with proline N-terminus gave a bright blue colour. This stain could be used before ninhydrin.

Carbohydrate. The paper was sprayed with 1% O-dianisidine in acetone. Dianisidine is carcinogenic and appropriate precautions were taken. On drying and strong heating, glucose samples gave a yellow/brown colour. This stain can be used before ninhydrin, which then gives green spots instead of blue.

Peptide Mobilities

The standards used in electrophoresis and chromatography were used to express the mobilities of peptides. Mobilities are expressed relative to lysine (m = + 1.0) and aspartic acid (m = - 1.0) and are measured from the centre of the neutral band (which moves slightly towards the cathode due to endosmosis) to the leading edge of the peptide band (Ambler, 1963b; Offord, 1966).

Figure 14 shows a plot of log (molecular weight) vs $\pm$ (mobility) at pH 6.5 for peptides with various charges (Offord, 1966). This figure was used to calculate the charge on a peptide from its mobility and amino-acid composition and hence the number of amides of acidic amino-acids could be calculated.

Following the detection of peptides on the guide strips of

electrophoretograms and chromatograms, the corresponding lateral strips were cut out and eluted into acid-cleaned test-tubes with 0.1 M-ammonia, or, for very big peptides, with 1 M-acetic acid.

Criteria of Purity

Peptides judged pure by paper electrophoresis and chromatography were analysed qualitatively for amino-acids and for N-terminal amino-acids. These analyses served as a further check of purity before committing the peptides to further analysis. The quantitative amino-acid composition gave a definitive assay of purity.

Peptide Nomenclature

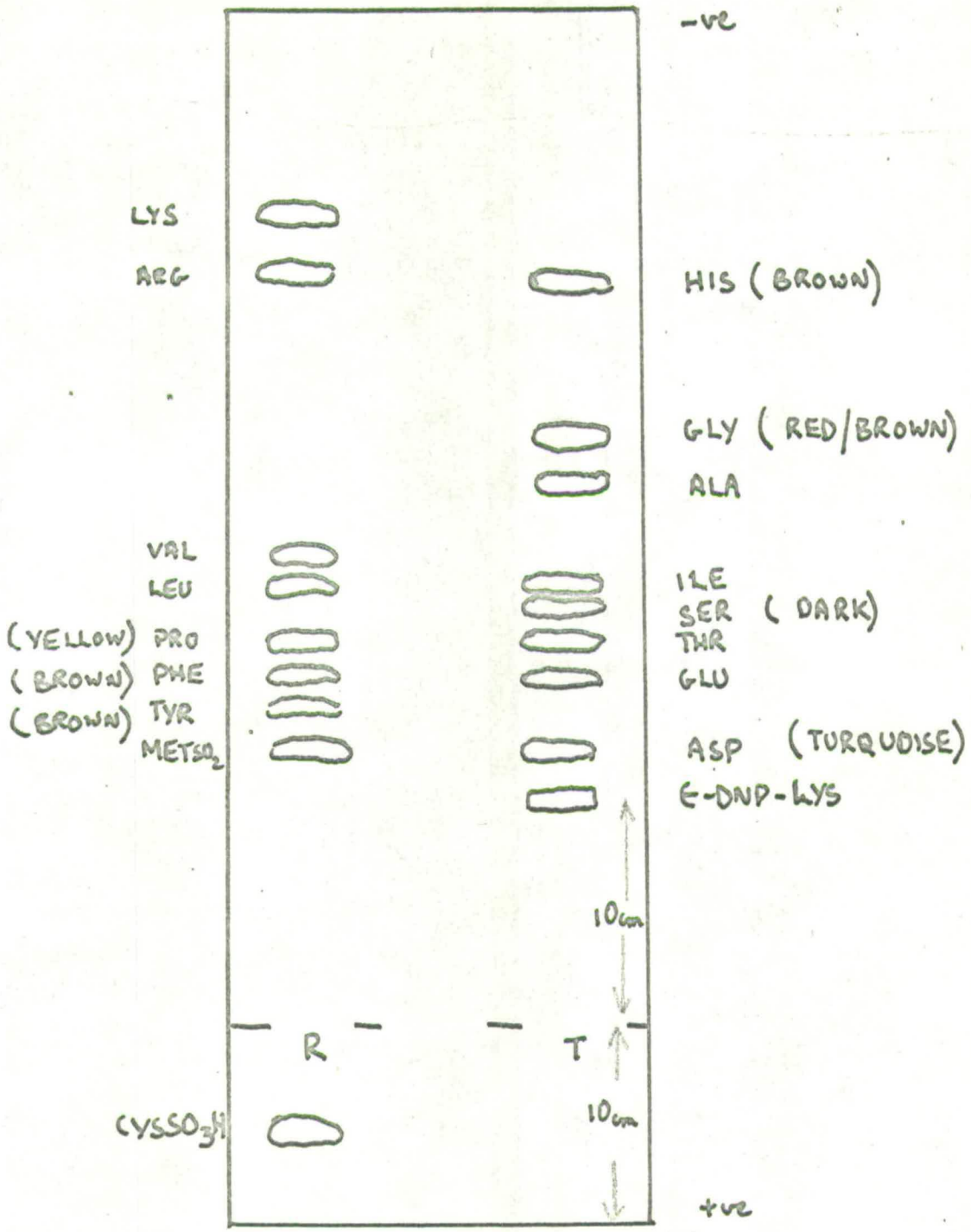
The initial letter in the designation of a peptide indicates the mode of digestion; T for trypsin, C for chymotrypsin, P for pepsin, X for cyanogen bromide, S for subtilisin, H for thermolysin and Q for papain.

Each of the primary fractions was numbered. Subsequent fractionation was numbered or lettered. Thus T17a, T17b and T17c were purified by electrophoresis from fraction 17 of an ion exchange purification of a tryptic digest. Similarly, C392a and C392b were found in fraction 3 of a gel-filtration purification of a chymotryptic digest and were subsequently purified by three different stages.

C. Analysis of Peptides

Composition

A qualitative determination of amino-acid composition was by



pH 2·0 ELECTROPHORESIS OF AMINO ACID MARKERS 5kV 15MIN.

FIGURE 15.

hydrolysis of a small sample of peptide followed by electrophoresis at pH 2.0 to separate the amino-acids (Levy and Chung, 1954). This technique was also used to check the purity of samples, to estimate how much of the samples to use in subsequent procedures and as a semi-quantitative analysis of small peptides produced by sub-digestion.

0.01-0.02 μmole of peptides were desiccated in test tubes (3.5 cm x 0.6 cm), to each of which was added 0.10 ml of 6N-HCl. The tubes were sealed and incubated overnight at 105°C. They were then opened and dried over NaOH *in vacuo*. Each sample was re-dissolved in 0.01 ml of 0.1 M-ammonia and applied as a 1 cm streak on a sheet of Whatman No.1 paper. The layout of the sheet, together with the composition and eventual disposition of the amino-acid markers is shown in Figure 15. Electrophoresis was at pH 2.0 at 5 kV for about 15 min, during which time the ϵ-DNP-lys marker moved about 10 cm. The paper was dried and dipped through 0.2% ninhydrin in acetone containing a few drops of glacial acetic acid and of 2,4,6-collidine (Midland Tar Distillers, Four Ashes, Staffs.). On gentle heating, aspartic acid appeared as a turquoise colour which deepened with stronger heating. The various colours of the amino-acids are shown in Figure 15. A tryptophan degradation product could sometimes be seen as a fluorescent spot near glutamic acid before staining.

For quantitative amino-acid analysis, samples were dried down in 10 x 1.2 cm Pyrex test tubes. 0.2 ml of concentrated HCl (BDH "Aristar") was added to each, followed by 0.2 ml of water. The tubes were drawn out, frozen in methanol/solid CO_2, evacuated to at least 0.2 torr and sealed. They were kept at 105°C for 24 h

and desiccated over NaOH. Threonine and serine are slowly destroyed by this treatment and so whole protein samples were hydrolysed for different times to permit an extrapolation to estimate the original amounts of these amino-acids (see Table 5 in Chapter II). Hydrolysates were normally stored *in vacuo* until immediately before analysis.

Alkaline hydrolysis with baryta water (Noltman *et al.*, 1962) was used to estimate tryptophan and methionine destruction in photo-oxidation experiments, but the hydrolysates were non-stoichiometric in most amino-acids, perhaps due to their absorption on to the solid barium carbonate.

Automatic amino-acid analysis was carried out by the accelerated two-column method (Benson and Patterson, 1965) on a Beckman Model 120C analyser or by an accelerated and modified version of the single-column method (Hamilton, 1963) on a Biocal BC200 analyser.

Samples were dissolved in 0.2 M sodium citrate pH 2.2 containing known amounts of norleucine and 2-amino-3-guanidopropionic acid as internal standards (Walsh and Brown, 1962). This permitted normalisation of results to minimise volumetric errors.

The analysis involves the separation of the amino-acids by chromatography on a cation exchange resin developed at high pressure by a series of buffers, followed by a continuous reaction of the effluent with ninhydrin in a boiling water bath and continuous automatic spectrophotometric monitoring of the product. Thus a chart is produced in which an amino-acid is characterised by the position, and quantitated by the area, of a peak (Spackman *et al.*, 1958).

Periodically, the analysers were calibrated by analysing

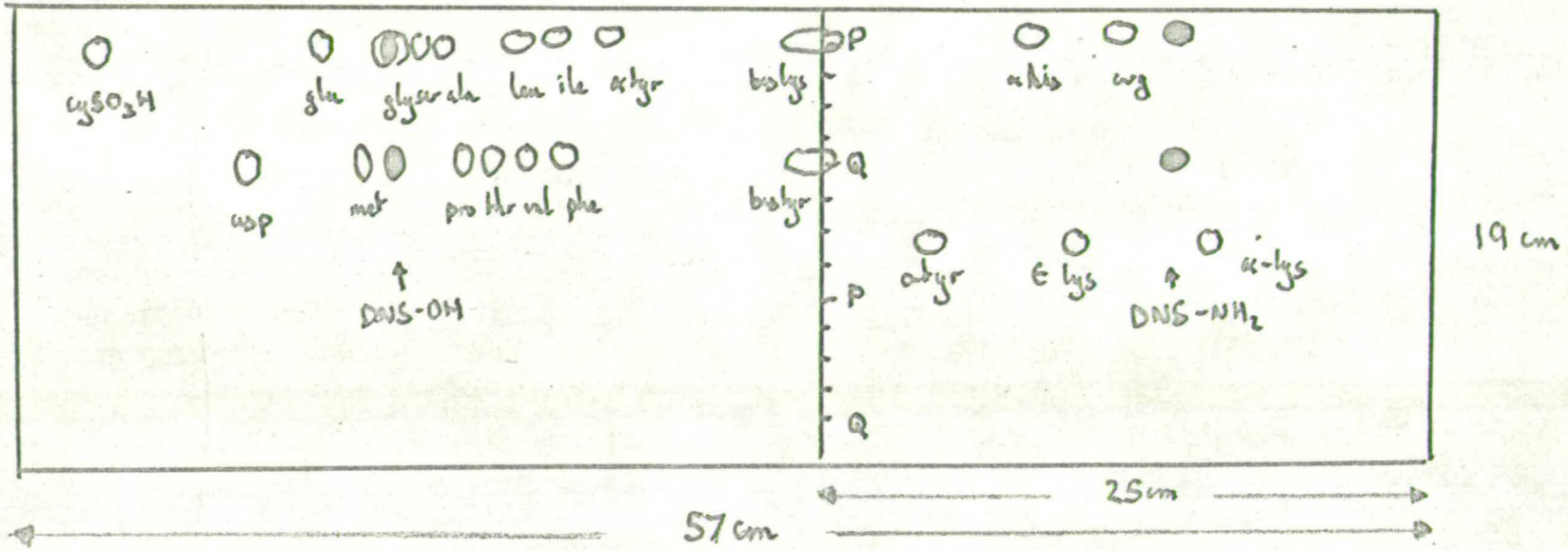
several standard amino-acid mixtures and thus calculating the "ninhydrin colour constants" which related the height of each peak to the amount of amino-acid present.

The accuracy and precision of the technique have been quoted as 100 ± 3% (Benson and Patterson, 1965) for samples of 0.05 to 0.2 μmole. The standard deviation on four successive standard single-column runs with 0.025 μmole of each amino-acid was 100 ± 3% and so the accuracy was 100 ± 6% (Spackman et al., 1958). Many peptide samples were of 0.010 μmole or less. When the indices of precision are compiled for the analysis, the normalisation of the analysis and the calculation of the amounts of amino-acid, a figure of 100 ± 9% emerges.

The results of peptide analysis are expressed in moles/mole of peptide to one decimal place and all impurities greater than 10% are included.

No amino-sugars were detected on whole protein analysis. Carboxymethylcysteine and 1- or 3-carboxymethylhistidine were detected by their position relative to other amino-acids and were estimated using the constants for aspartic acid and glutamic acid respectively (Gurd, 1967). 3-nitrotyrosine was quantitated in the normal way, using a standard solution.

CNBr peptide hydrolysates contained almost equal amounts of homoserine lactone and homoserine. If the hydrolysates were heated to 105°C for 1 hr in a sealed tube with 0.1 ml of pH 6.5 electrophoresis buffer, dried down and re-dissolved immediately before application to the analyser, then the lactone was converted completely to homoserine and could be separated from glutamic acid on a column of Locarte No.12 resin at pH 2.87 (Ambler, 1965).



pH 4.38 ELECTROPHORESIS OF DNS-AMINO ACIDS 7kV 2 HR.

FIGURE 16.

This procedure was followed for analysis of the major CNBr peptides. Homoserine was not resolved on the single column analysis and even long incubations in anhydrous TFA before analysis did not give complete recovery of homoserine lactone (Armstrong, 1949). For the analysis of sub-peptides by the single-column system, no attempt was made to alter the equilibrium.

Peptide N-termini (Gray 1972a)

0.01 - 0.02 μmole samples of peptide were dried down in small (2.5 cm x 0.4 cm) test tubes, 0.01 ml of 0.1 M-sodium bicarbonate was added to each and this was also dried down. 0.01 ml of water followed by 0.01 ml of Dns-Cl in acetone (2.5 mg/ml) was then added. The tubes were sealed in small beakers with "Parafilm" and incubated at 37°C for 1 h. They were then dried down, 0.050 ml of 6N-HCl was added, the tubes were sealed and incubated overnight at 105°C (4-6 h if proline was suspected at the N-terminus). When cool, they were open and dried down over NaOH.

The samples were taken up in 0.01 ml of 50% pyridine and applied to a sheet of Whatman 3 mm paper for electrophoresis at pH 4.38 (Pyridine:Acetic Acid:Water :: 3:6:500). The dimensions and layout of the paper, together with the eventual positions of the Dns-amino-acid markers, is shown in Figure 16. The electrophoresis was in an apparatus of the "cooled-plate" type (Gross, 1961) with a potential difference of 7 kV and a current of 170-220 ma for 2 h. Best results were obtained with pH 4.38 pyridine acetate buffer in the reservoirs and pH 4.40 buffer for wetting the paper. Dns-tryptophan and α-Dns-tyrosine were rarely detected by this technique but bis-Dns-tyrosine signified that tyrosine was N-terminal.

Dns-ser, Dns-ala and Dns-gly were cut out of the paper and sewn on to a sheet of Whatman 3 mm for electrophoresis at pH 2.0 (Naughton and Hagiopan, 1962). Similarly Dns-leu, Dns-val, Dns-ile, Dns-phe, Dns-pro and Dns-thr were confirmed by descending paper chromatography in PEAW (Boulton and Bush, 1964). The chromatogram was placed in the tank for 2 h with a "balance sheet" to saturate the inside of the tank with vapour before the 16 h chromatography was started.

Peptide C-termini

The carboxypeptidase A and B were prepared as described in Chapter III. 0.025-0.05 μmole of peptide in the appropriate buffer were reacted with 0.005-0.10 ml of the enzyme for a given time. The amino-acids released were sometimes identified by electrophoresis and more often on the amino-acid analyser.

Peptide Sub-digestion

The trypsin and chymotrypsin solutions described earlier, diluted to 1 mg/ml and pepsin freshly made up at 1 mg/ml, were used for peptide sub-digestion. The conditions were as for protein digestion, with 0.01 ml of enzyme for 0.1 μmole of peptide.

Subtilisin (Novo) was dissolved in 0.2 M-ammonium acetate pH 8.5 at 1 mg/ml and the peptide was dissolved in the same buffer.

Papain (Sigma; 17 mg/ml suspension in water) was added to the peptide dissolved in 0.1 ml pyridine acetate pH 6.5 (electrophoresis buffer), containing 0.001 M dithiothreitol. 0.010 ml of suspension was used with approximately 0.2 μmole of peptide. The tube was flushed with nitrogen, sealed and incubated for 10 h at 37°C (Ambler, 1963b).

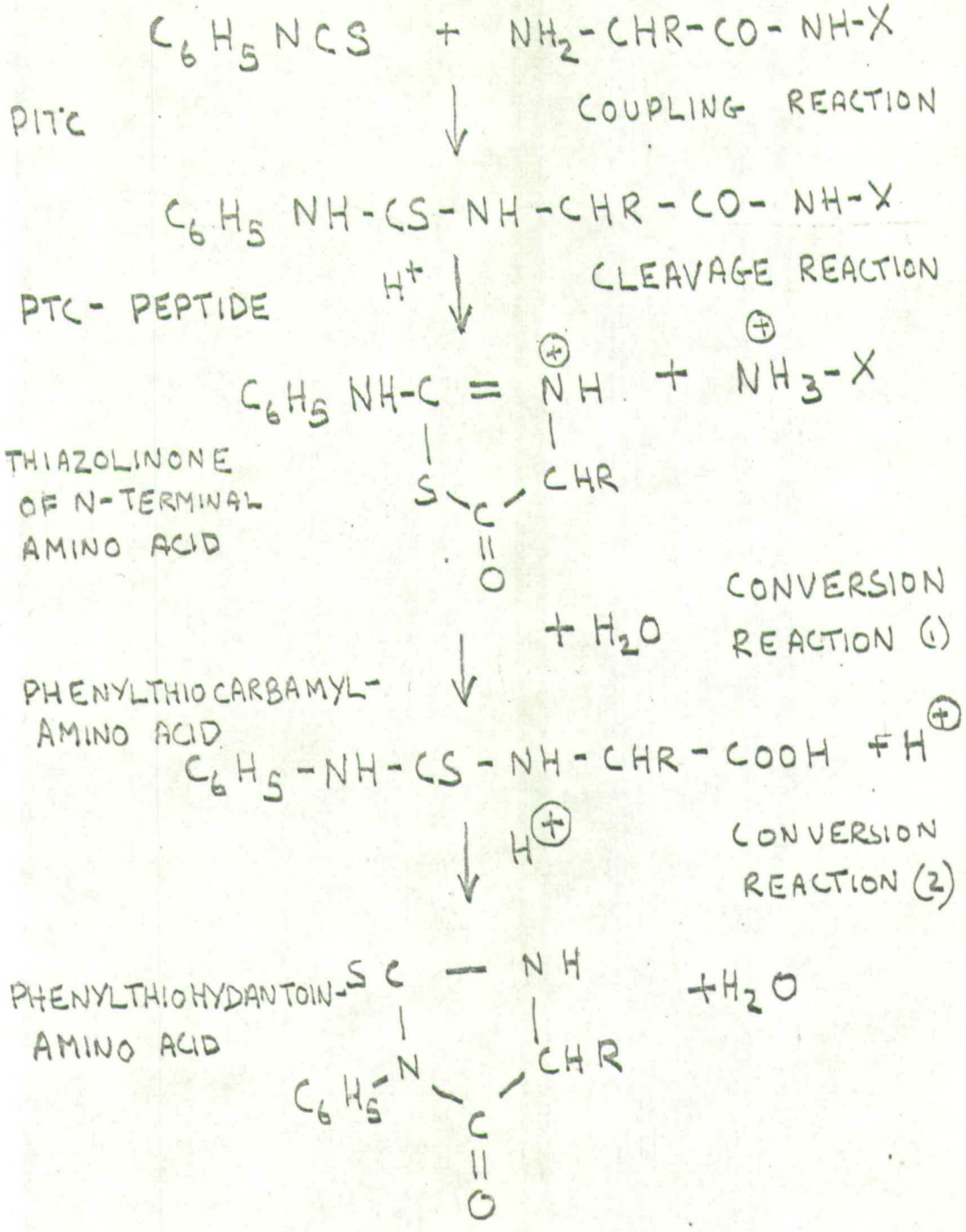


FIGURE 17 THE EDMAN REACTION

Thermoysin (Daiwa Kasei, Osaka, Japan) was dissolved at 0.5 mg/ml in 0.2 M ammonium acetate pH 8.5, 0.005 M in $CaCl_2$ and 0.01 ml was incubated with 0.1-0.2 μmole of peptide, dissolved in the same buffer, for 3-4 h (Ambler and Meadway, 1968).

After sub-digestion, the solutions were dried down in a desiccator and then fractionated, usually by pH 6.5 or pH 3.5 electrophoresis. A strong neutral band observed at pH 6.5 was cut out and sewn on to a paper for electrophoresis at pH 3.5.

The Edman Reaction (Figure 17)

Chemical sequencing was done by Edman's reaction (reviewed by Edman, 1970) using the "Dansyl-Edman" procedure (Gray, 1972b). Peptide samples were dried down in small screw-capped tubes, and then redissolved in 0.20 ml of 50% pyridine. 0.10 ml of phenylisothiocyanate (5% in pyridine) was added and the tubes were flushed with nitrogen, sealed and incubated at 37°C for 1 h. The PITC solution was stored at -10°C under nitrogen and was freshly prepared every two or three days.

The tubes were desiccated at 60°C for 1 h and, when cool, 0.15 ml of anhydrous trifluoroacetic acid was added. They were flushed with nitrogen, sealed and incubated at 37°C for 0.5 h and then dried down in a warm desiccator for 0.5 h.

0.25 ml of water was added, followed by 1 ml of butyl acetate. The tubes were mixed thoroughly and the butyl acetate layer was separated by centrifugation and discarded. This extraction was repeated twice and the samples were then dried down prior to the next cycle.

For the second and subsequent cycles, the samples were redissolved in more than 0.20 ml of 50% pyridine, such that a suitable

portion could be removed for N-terminal analysis. Starting with 0.2 μmole of an octapeptide, one-eighth of the material after the first cycle was removed. This fraction was increased until one-half was removed prior to the last cycle. In some cases the last amino-acid was identified by pH 2.0 electrophoresis but often, especially with small amounts of larger peptides, the chemical sequencing was incomplete.

The use of fresh PITC was critical, as was the necessity of a nitrogen flush before the coupling reaction between PITC and the N-terminus of the peptide, as oxidative desulphuration of the PTC-amino-acid can occur, leading to a phenylcarbamyl compound which will not react further (Edman, 1970). Thus some or all of the peptide sample can be effectively lost.

Small bottles of TFA were used as it is hygroscopic and aqueous TFA might lead to peptide bond cleavage.

The extraction procedure was sometimes omitted towards the end of a degradation where low yields were suspected. Sometimes no portions were taken for the first few cycles, if the peptide had already been partly sequenced.

"Raggedness" i.e. the sequential degradation becomes partly out of phase and several N-termini are identified after each cycle, was encountered in some cases. At the cleavage reaction, the equilibrium does not lie entirely towards the free PTC-amino-acid and so there is some residual PTC-peptide which may be cleaved at the next cycle (Edman, 1970). Automatic sequential degradation incorporates two cleavage reaction steps to avoid this effect.

SPITC

One of the disadvantages of the Edman degradation is that a large, denatured polypeptide, which is relatively insoluble in the reagents used, is rendered even less soluble by the formation of hydrophobic ϵ-PTC-lysine derivatives. The recent availability of 4-sulpho-PITC (SPITC; Pierce; Birr et al,, 1970) prompted some experiments with this compound as a means of rendering polypeptides soluble throughout the Edman degradation.

Preliminary experiments indicated that in 50% pyridine, SPITC reacted very slowly with ribonuclease. The pH of 50% pyridine was found to be about 7 and so 0.4 M N-ethyl morpholine in 50% pyridine, adjusted to pH 9.3 with 25% aqueous TFA was used as the buffer for this reaction.

The reaction of SPITC with ribonuclease was followed by assaying free amino groups with 2,4,6-trinitrobenzene sulphonic acid (Habeeb, 1966). 1 μmole of oxidised ribonuclease was dissolved in 1.0 ml of the buffer described above and divided into 4 aliquots, to each of which was added 0.1 ml of 7% SPITC. The mixtures were incubated at 37°C for different times, from 2-14 h after which 0.1 ml of 0.1% TNBS in 0.1 M-$NaHCO_3$ buffer pH 9.3 was added, followed by a further incubation at 37°C for 1 h, when 0.1 ml of 10% SDS and 0.05 ml of 1 M-HCl was added. After standing for 5 min, 1.0 ml of water was added and the extinction at 335 nm was measured, relative to a reagent blank. It was calculated that a 2 h incubation with SPITC blocked 45%, a 6 h incubation blocked 80%, and a 14 h incubation blocked 91% of the amino groups.

SPITC was dissolved at 70 mg/ml in 0.4 M NEM in 50% pyridine, pH 9.3 and 0.10 ml was added to 0.2 μmole protein in 0.20 ml of the

Table 9.

Sequential Degradation of 0.25 μmol SPITC-Treated Ribonuclease

Cycle	1	2	3	4+	5	6	7	8
lys	.012	.006	.006				.010	.002
asp						.009	.009	.001
thr								
glu		.020	.004			.007	.008	.001
gly	.005		.008			.007	.008	.004
ala	.005				.010	.017	.013	.003
val	.004					.003		
ile	.004	.002	.002			.001	.002	
leu	.006	.002	.008			.001	.003	
tyr		.007						
phe								.004
% Yield	5	8	3.5	-	4	7.5	5	1.5
Sequence from these results	lys	glu	*		ala	ala	ala	phe/gly
Actual sequence 1	lys	glu	thr	ala	ala	ala	lys	phe

* PTH-thr undergoes β-elimination on hydrolysis to yield gly (Van Orden and Carpenter, 1964).

\+ Faulty analysis.

1 Smyth et al., 1963.

same buffer. The tube was flushed with nitrogen, sealed and incubated, with shaking, for 18 h at 37°C. The solution was then desalted on Sephadex G-25 in 0.1 M-ammonia, to remove excess SPITC, and was freeze-dried.

The SPITC-protein was degraded by the Edman method, using the "SDS-Dansyl" technique (K. Weber, unpublished; Chapter II) for the identification of the N-terminus after each stage. Residues 2-6 in bovine ribonuclease and residues 2-5 in sperm whale myoglobin (Dayhoff and Eck, 1970) were successfully identified in this way. Experiments with β-lactoglobulin, deoxyribonuclease and β-lactamase were unsuccessful.

SPITC-ribonuclease was also sequentially degraded as described above, but with extraction with ethyl acetate at the end of each cycle instead of butyl acetate. The pooled ethyl acetate was removed in a stream of N_2 and the residual PTC-amino-acid was desiccated and then hydrolysed in 6 M-HCl *in vacuo* at 130° for 24 h (Van Orden and Carpenter, 1964). The HCl was removed over NaOH *in vacuo* and the solid was dissolved in 0.2 M-sodium citrate pH 2.2 and analysed for amino-acids. Table 9 shows the results of this experiment. The results of similar experiments with other proteins were poor, probably due to faulty extractions which led to contamination of the hydrolysates with whole protein. With care, the technique may be useful for sequencing the first few residues in proteins and large peptides.

SPITC and other hydrophilic PITC derivatives have been successfully employed in automatic sequential degradation (Braunitzer *et al.*, 1971).

CHAPTER V.

TRYPTIC PEPTIDES OF *E.COLI* β-LACTAMASE

In the course of this work, five independent tryptic digests have been examined; the experimental details relating to them are summarised in Table 8 (Chapter IV). Figure 19 shows the peptide map of the fractionation of the second tryptic digest by cation-exchange chromatography; this procedure was also followed with the third and fifth digests. The fourth digest was initially fractionated by gel filtration on Sephadex G-25 in 5% formic acid, in order to ensure that peptides were not irreversibly bound to the ion-exchange resin. The first digest was fractionated in the same way; the yields of peptides were very small and have not been included in the following results.

Table 10 lists tryptic peptides which have been found in good yield and extensively characterised. Table 11 lists tryptic peptides for which analytical data is less reliable. Table 12 lists some peptides found in low yield in tryptic digests. Peptides for which amino-acid analyses were uninterpretable have not been included.

The electrophoretic mobilities (defined in Chapter IV) were mainly measured on preparative electrophoresis. V/Vo was not accurately determined and so the numbers in these columns represent the primary fraction(s) in which the corresponding peptides were found in the fourth digest.

The Tryptic Peptides

1. T2d/T4b — arg,asx,thr_4,pro,ala_3,met_2,leu

These peptides have the same composition and the same N-terminal

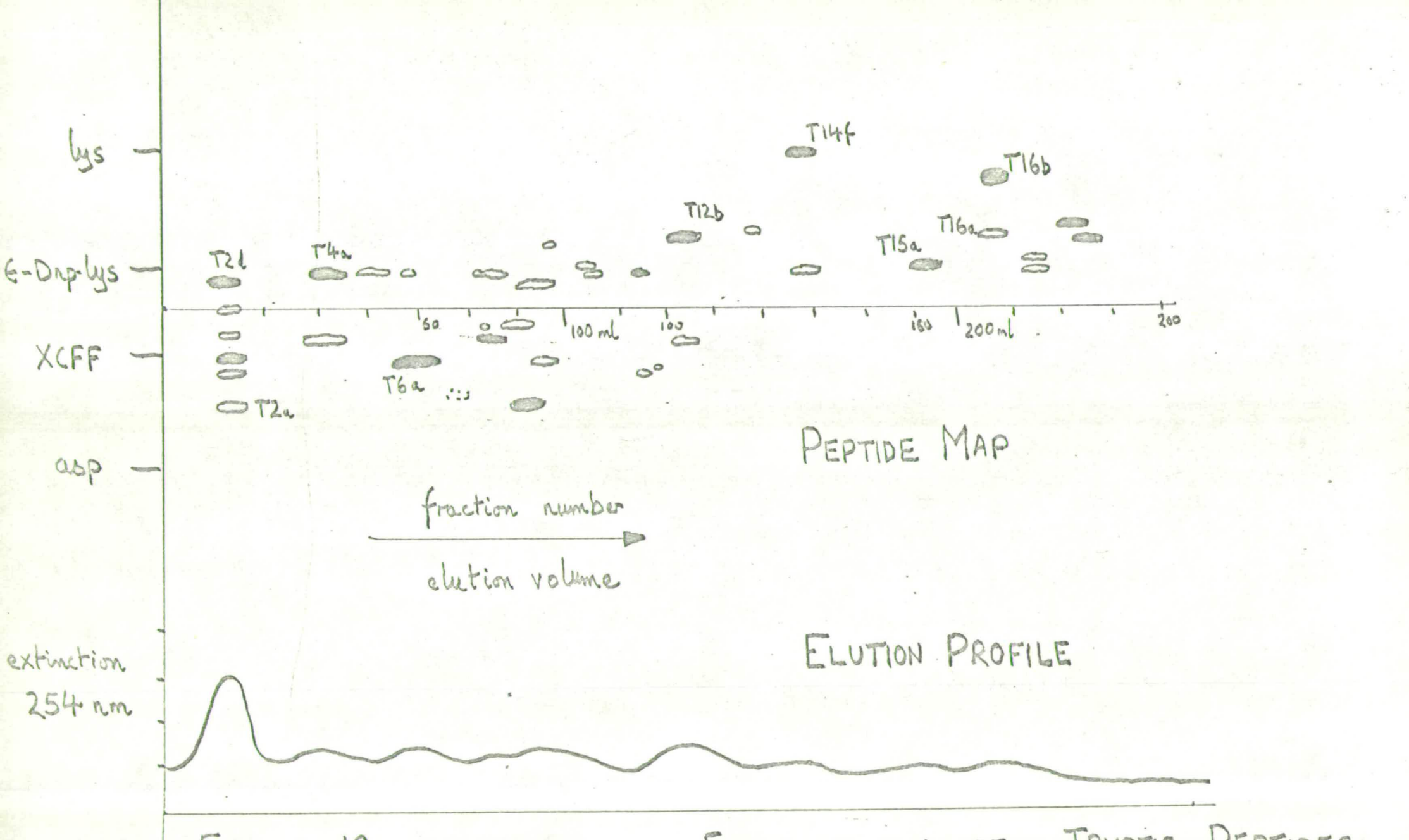


FIGURE 19 FRACTIONATION OF TRYPTIC PEPTIDES

Key To Table 10

The column "V/V_o G-25" simply lists the number of the Sephadex G-25 primary fraction in which the peptide was found; accurate elution volumes were not calculated.

The purification of a peptide is summarised by:

C : ion-exchange chromatography

S : Sephadex G-25 gel filtration

6 : electrophoresis at pH 6.5

3 : " " pH 3.5

2 : " " pH 2.0

B : paper chromatography in BAWP.

The symbols are arranged in the order in which the purification stages were carried out.

Some amino-acid analyses are reported as estimated residues per peptide. This was sometimes necessary with bad amino-acid analyser runs. Others are reported to one decimal place in residues per peptide, with all values greater than 0.1 included. Tryptophan was usually detected by Ehrlich's reagent on paper, but not quantitatively analysed.

Unequivocal N-termini and sequential degradation results are reported. Tentative identifications and weak spots of Dns-amino-acids are bracketed. Complete sequential degradations are terminated by a vertical bar.

This key also applies to Tables 11, 12, 14, 15, 17, 19 and 20.

TABLE 10 TRYPTIC PEPTIDES

S : purification by Sephadex G-25
C : " by ion-exchange chromatography
6, 3, 2, B : " by electrophoresis and paper chromatography

Peptide	m 6.5	V/Vo G-25	Putn	Yield %	Gly	Ala	Val	Leu	Ile	Ser	Thr	Asp	Glu	Phe	Tyr	Trp	Cys	Met	Pro	Lys	His	Arg	TOT	N-t	-1	-2	-3	-4	-5	-6	-7	-8	-9
T2d	-0·05	3	C63	2-8	0·2	3·0		1·0			3·7	1·0	0·1					1·9	1·0			1·0	13	asx	thr	thr	met	pro	ala	ala	met	—	
T4b	0·0	—	C632	3	0·3	2·9	0·2	1·0	0·2	0·4	3·7	1·1	0·5					1·7	1·2	0·1		0·9	13	asx	—	thr	met	(pro)	—				
T6a	-0·85	4	C6	2		1·0		0·2	0·1			2·1	1·3							1·0			5	asx	ala	glx	asx	lys					
T9a	-0·25	—	C63	5		1·0						2·2	1·3							1·0			5	asx	ala	(glx)	—						
T15a	-0·25	3	S63	5		1·0	0·9					2·1	1·1							2·3			7	val	lys	asx	ala	glx	—				
T18d	0·95	5	S6	2			0·9													1·0			2	val	—								
T6b	-0·25	3/4	C63	11	1·9	1·0	1·0	1·0	0·1	0·1	0·1	1·0	3·0									0·9	10	val	asx	ala	gly	glx	—				
T8c	-0·1	—	C633	3	1·9		1·0	2·2	1·1	0·9		2·0	1·0		0·9					1·1			12	val	tyr	—							
T11a	-0·5	2/3	C62	2-5	0·2	1·1		1·8	0·9			4·1	4·4						1·7			2·1	16	leu	asx	arg	glx	glx	(glx)	—			
T15b	0·0	4/5	C633	8	0·1	0·1		1·0		0·2	0·1	1·0	0·1									1·0	3	leu	asp	arg							
T11b	0·0	3	C62	3	1·1	3·2		1·1	1·1	0·9		1·0		1·0		++			1·0	1·0			12	ser	ala	LVIF	ala	—					
T12b	0·4	3	C63	1-10	3·2	2·2	0·3	1·0	1·1	1·7		1·1							2·0	1·1		1·0	14	gly	leu	ile	(ala)	(pro)	(gly)	—			
T14a	0·0	4	C63	8-16	2·0	1·0		0·1	0·1	0·8		0·1	1·0									1·2	6	ser	gly	ala	glu	gly	arg				
T14d	0·45	-	C63	4	0·1	0·1				1·0	1·0		0·1	1·7				1·8	1·0	0·9			8	phe	(pro)	met	met	(ser)	—				
T14e	0·5	1	S63	5	0·1			0·1	0·1	1·0	1·1			2·0				1·8	1·1	1·7			9	phe	—	—	met	ser	(thr)	phe	lys	—	
T14f	1·0	5	C6	8																1·0			1	lys									
T16b	0·9	5	C6	5																		1·0	1	arg									
T19b	1·05	5	S6	—																1·0			1	lys									
T17a	0·1	4	C32	5	0·1	0·1		1·0	1·0	1·0	0·1	0·1	3·4	0·9					1·0			2·0	10	phe	ile	glx	arg	(pro)	—				
T17b	0·1	3	C63	9	1·1		1·1	1·2			2·0	1·0	0·3					1·0			0·8	1·0	9	his	leu/val	asx	thr	gly	met	thr	—		
T17c	0·15	3/4	C63	14	0·2	0·1	1·1	1·1	0·2	0·3	1·0		1·1						1·0	1·0	0·7	0·3	7	his	glu	thr	pro	leu	val	lys			
T18a	0·2	4	C63	10	1·0	1·0		0·8														1·1	4	leu	gly	ala	arg						
T18b	0·6	4	C63	5	1·0					1·0		0·1	0·1									0·9	3	gly	ser	arg							
T18c	0·85	5	S63	5	0·1	0·1		0·1	0·1	0·2		1·0										0·9	2	asn	arg								

TABLE II TRYPTIC PEPTIDES

Peptide	m 6.5	V/Vo G-25	Purn	Yield %	Gly	Ala	Val	Leu	Ile	Ser	Thr	Asp	Glu	Phe	Tyr	Trp	Cys	Met	Pro	Lys	His	Arg	TOT	N-t	-1	-2	-3	-4	-5	-6	-7	-8	-9
T2a	-0.65	(2)	C6	8	0.2	1.0	0.2	1.5	1.3	0.4	1.0	3.7	3.2	0.7			0.8	1.0	1.0	1.1		0.3	16	asx	gly	pro	cys	glx	glx	ile	(phe)	—	
T2b	-0.3	—	C6	8	2.0	2.0	1.2	2.7	1.1	1.0	2.9	5.1	2.8					0.3	1.7	1.0		0.3	24	asx	glx	asx	Thr	LVIF	asx	leu	(strongest spots)		
T3	-0.1	—	C63	5	1.0	2.3	0.4	2.1	2.0	1.0	1.5	1.9	3.1					0.8		1.0			16	asx	—								
T6d	-0.2	—	C63	2	1	4	1	3	1	1	2	3	6	1				1		1		ND	25	glx									
T7	0.0	—	C63	5	1	3	1	3	3	1	1	5	7		1	++		1	3	1		2	34	—									
T12a	-0.2		C683	2	0.6	0.6	2.1	1.6	1.0	1.8	0.9	2.5	3.6			++			1.0	1.5	0.9		—	—									
T16a	0.2	2/3	S632	2-5	1.1	0.8	1.3	2.2		0.3	2.2	2.0	1.9	0.6				1.1			1.0	1.0	15	glx	leu	val	—						
T19a	0.0	5	S63	1						1.0													1	ser									
T19c	0.0	5	S63	—				1					1										(2)	—									

TABLE 12 TRYPTIC PEPTIDES

Peptide	m 6.5	V/Vo G-25	Purn	Yield %	Gly	Ala	Val	Leu	Ile	Ser	Thr	Asp	Glu	Phe	Tyr	Trp	Cys	Met	Pro	Lys	His	Arg	TOT	N–t
T4a	-0·1	–	C63	0·5	1	2	1	2	1	4	1	2	2							1			17	ser
T4c	0·05	–	C63	1	0·4	1·2	0·1	1·0	1·0	0·5	0·2	2·0	3·2					0·8		1·0			10	–
T5c	0·0	–	C3	–	0·8	1·0	0·3	0·4	0·2	0·4								0·3		0·1			–	–
T9b	0·0	–	C63	1	3·3	1·2	1·1	6·0	1·2	2·0	1·8	2·3	2·4	0·8				1·0		1·0		0·9	24	–
T11c	0·0	4	C62	0·5		1·5				1·0		1·0	1·2							1·1			5	ser
T14b	0·19	–		0·5	1·0	1·2	1·0	2·1		0·1								0·8				1·1	7	val
T14c	0·3	–		0·5						0·2		0·9	1·0						1·0	1·1			4	asx
T145	-0·1	–	C36	0·5						0·9			2·0									1·0	4	–
T28	0·4	2	G63	0·5	2	1	1	2															6	–
T38	0·5	3	G63	–	0·7	1·0		0·7	2·0				1·0							1·1			7	–
T34	-0·5	3	G62	1	1	1		1		1	1	2	3							1			11	—

sequence. Peptide T4b was isolated in lower yields than T2d in the second and third digests but this may have been due to the more extensive purification that was necessary. The electrophoretic mobilities of these peptides are:

	m	m'
T2d	-0.05	0.2
T4b	0.0	0.1

They are effectively neutral at pH 6.5 and have no amide groups but T4b is slightly more acidic at pH 3.5. A possible explanation of this phenomenon is that T2d contains α-aspartic acid and that T4b contains β-aspartic acid. An equilibrium between the two forms could be set up in the acid conditions prevailing during the oxidation of β-lactamase and the fractionation of the products (Swallow and Abraham, 1958). The relative mobilities at pH 3.5 are comparable with those of α- and β-aspartyl-peptides (Swallow and Abraham, 1958; Naughton et al., 1960). An equilibrium between the two forms would explain why a partial sequential degradation was possible.

Peptide 2d was sub-digested with chymotrypsin. Most of the compositional data is qualitative.

Peptide	m'	N-terminus	Composition
T2dc1	-0.36	asx	asp,thr_2,met_2,pro,ala_2
T2dc2	-0.1	asx	(asp,thr,met,pro,ala)
T2dc3	+0.25	ala	(ala,thr,leu)
T2dc4	+0.5	ala	(ala,thr,leu,arg)
T2dc5	+0.65	-	-
T2dc6	+0.9	arg	(arg)

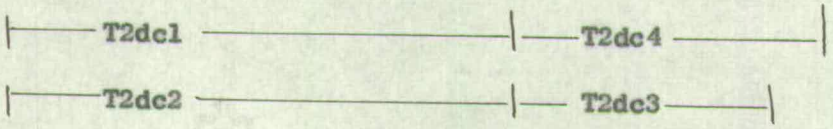
The sequence of T2dc4 was ala-thr-thr-(leu,arg). T2dc1 and T2dc2

may represent α- and β-aspartyl-isomers of the N-terminal chymotryptic peptide.

Incubation of T2d with CPA and CPB (0.07 μmol peptide, 0.02 ml each enzyme) released the following amino-acids: thr, 1.8; ala, 1.0; met, 0.4; leu, 1.0; arg, 1.1.

The sequence of T2d is:

asp-thr-thr-met-pro-ala-ala-met-ala-thr-thr-leu-arg



2. T6a/T9a asx_2,glx,ala,lys

T6a and T9a have identical compositions except that T6a has no amides and T9a has one. T9a was found in higher yields despite lengthier purifications. It gave no further results after two cycles of the Edman degradation despite a sufficiency of material; this may have been due to the formation of pytoglutamic acid from glutamine (Blombäck,1967; Edman, 1970). Glutamine is deamidated relatively easily at low pH (Wilson and Cannan, 1937) and so T9a is probably represented in the sequence and T6a is probably derived from it.

The sequence of T9a is:

asp-ala-gln-asp-lys

3. T15a asx_2,glx,ala,val,lys_2

T15a has no amides, but from its composition and sequence is

clearly related to T9a.

val-lys-asp-ala-glu-(asp,lys)

4. T6b arg,asx,glx_3,gly_2,ala,val,leu

T6b has two amides. It was sub-digested with subtilisin.

Peptide	m	N-terminus	Composition
T6bs1	-1.0	val	(asx,glx,val,ala,gly)
T6bs2	0.0	glx	(glx,leu)
T6bs3	+0.65	glx	(glx,leu,gly,arg)
T6bs4	+1.0	-	-

The ninhydrin stain for each of these peptides was very weak. From the mobilities, T6bs1 probably contains asp and glu, and T6bs2 and T6bs3 both contain gln.

CPA and CPB released amino acids tentatively identified as arg, gly and gln, by paper electrophoresis.

The following sequence is tentatively deduced:

val-asp-ala-gly-glu-gln-leu-(gln,gly)-arg

T6bs1

T6bs2

T6bs3

5. Peptide T6c (m = -0.1 approx) was isolated in low yield and had a similar qualitative composition to T6b.

6. T8c lys,asx_2,ser,glx,gly_2,val,ile,leu_2,tyr

This peptide has been isolated twice but has required further

purification after an initial analysis on both occasions. One attempt at sequential degradation stopped after two cycles for want of material and in another no results were obtained, possibly due to the use of an old PITC solution.

A single, neutral peptide T8ccl was isolated from a chymotryptic sub-digest, was analysed, and corresponded to asx-(asx,ser, gly,leu,lys). The residual peptide would have a hydrophobic N-terminal residue and no -amino groups and so might react poorly with ninhydrin. As the stain for ninhydrin-negative peptides was not used, it was not detected.

T8c probably has two amides and T8ccl probably has one. The partial sequence of T8c is:

val-(gln,gly,ile,leu,tyr)-asx-(asx,ser,gly,leu)-lys

|——— T8ccl ———|

7. T11a arg_2,asx_4,glx_4,pro_2,ala,ile,leu_2

T11a was isolated in all tryptic digests but was very difficult to purify. In one case, at least, two or three peptides of approximately this composition were isolated. This phenomenon may have been partly due to deamination or α-, β-aspartyl isomerisation during the purification but was certainly aggravated by the fact that the peptide was present in two adjacent primary fractions produced by Sephadex G-25 gel filtration.

The number of amides was estimated as being three, but in such a large, acidic peptide, this is not a reliable value.

Three attempts at sequential degradation proceeded no further than 'glu'; from the strength of DNA-glu observed, an N-terminal

sequence leu-asx-arg-glu-gln or leu-asx-arg-glu-glu-gln is probable.

A thermolysin sub-digest was carried out. No ninhydrin-negative peptides were detected. The amino-acid compositions of the sub-peptides are qualitative.

Peptide	m	m'	N-terminus	Composition
T11ah1	0.0	0.07	pro	(glx,pro,leu/ile,asp)
T11ah2	0.0	0.1	-	(asx,glx,pro,leu/ile)
T11ah3	0.0	0.13	-	(glx,asx,leu/ile,ala,arg)
T11ah4	+0.3	-	ala	(asx,glx,leu,ile,ala,arg)

A tentative, partial sequence based upon this data is:

leu-asx-arg-glx-glx-pro-(pro,glx,asx_2,leu/ile)-ala-(asx,glx,leu/ile)-arg

T11ah3 — T11ah1 — T11ah4

T11ah2

8. T15b leu-asp-arg

This is the structure of T15b, based upon its composition, sequence and mobility. It is probably related to T11a. It was isolated in relatively high yield, in the second digest, which suggests that a peptide corresponding to the remainder of T11a should have been found. Despite a search for ninhydrin-negative peptides, this postulated peptide has not been found. In later digests, the yields of T11a were higher and those of T15b relatively much lower.

9. T11b (trp),lys,asx,ser,pro,gly,ala_3,leu,ile,phe

The presence of tryptophan in T11b is inferred from the UV

fluorescence of the peptide, the positive result with Ehrlich's reagent on paper and the pink colour during the cleavage stage of sequential degradation which has been seen with other tryptophan-containing peptides.

The partial sequence of T11b is:

ser-ala-L1F-ala-(trp,asx,pro,gly,ala,$L1F_2$)-lys

10. T12b lys,arg,asx,ser_2,pro_2,gly_3,ala_2,ile,leu

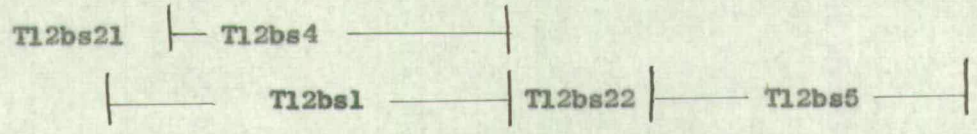
T12b probably has one amide. It was sub-digested with subtilisin. The compositions of sub-peptides were determined qualitatively.

Peptide	m	m'	N-terminus	Composition
T12bs1	-0.37	0.1	leu	(asx,pro,leu/ile,gly, ala)
T12bs21	0.0	0.27	gly/ala	(ser/leu/ile,gly)
T12bs2	0.0	0.35	(ser)	(ser/leu/ile,ala)
T12bs3	+0.4		gly	(asx,pro,ser,leu/ile, ala,gly,lys,arg)
T12bs4	+0.6		ile	(asp,leu/ile,ala,gly)
T12bs5	+0.8		gly	(pro,ser/leu/ile,gly, lys,arg)

CPA and CPB removed only arginine from this peptide.

The tentative, partial sequence based upon this data is:

gly-leu-ile-ala-pro-(gly,asn)-ser-ala-gly-ser-lys-pro-arg



The C-terminal sequence is based upon the composition and N-terminus of T12bs5, the fact that CPA and CPB will not remove C-terminal proline from peptides (Ambler, 1972) and the fact that trypsin does not normally hydrolyse a lys-pro bond (Hill, 1965). The latter assumption may be ill-founded since Milstein et al. (1968) have isolated a peptide produced by the tryptic cleavage of an arg-pro bond in a Bence-Jones protein.

11. T14a ser-gly-ala-glu-gly-arg

12. T14d $lys, met_2, thr, ser, pro, phe_2$

T14e $lys_2, met_2, thr, ser, pro, phe_2$

T14d and T14e differ in composition by a single lysine residue and are almost certainly homologous. They were not found in the same digests.

The N-terminal sequences established by sequential degradation are:

T14d phe-(pro)-met-met-(ser)-

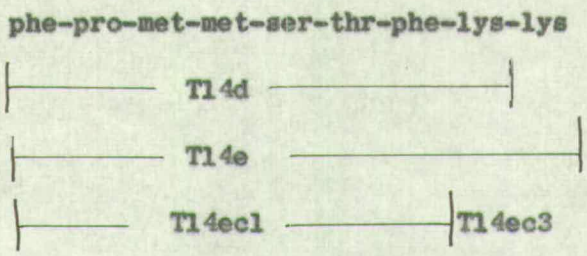
T14e phe - - met-ser-(thr)-phe-lys-

The amino-acids in parentheses were weak or tentatively identified.

T14e was sub-digested with chymotrypsin. The compositions of sub-peptides are qualitative.

Peptide	m	N-terminus	Composition
T14ec1	+0.13	phe	(met,pro,phe,thr,ser)
T14ec2	+0.4	Very weak, probably T14e	
T14e3	+1.0	lys	(lys)

The sequence which incorporates T14d and T14e is:



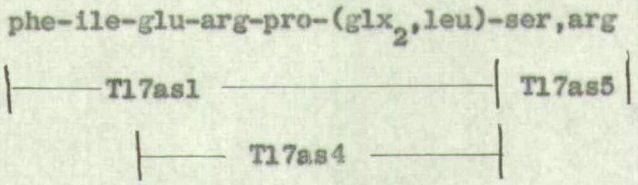
13. T14f and T16b are free lysine and free arginine respectively. They were not found when undigested β-lactamase is analysed (e.g. as controls for CPA experiments, Chapter III) and were therefore genuine products of digestion. Peptides T14d and T14e show how free lysine could be produced. Hydrolysis of N-terminal lysine and arginine is relatively slow (Hill, 1965). T19b (m = 1.05) contains only lysine and may represent a lys-lys dipeptide.

14. T17a arg_2,ser,glx_3,pro,leu,ile,phe

T17a has one amide. It was sub-digested with subtilisin. The compositional data is qualitative.

Peptide	m	m'	N-terminus	Composition
T17as1	-0.25		ile/phe	(phe,glx,ile/leu,arg)
T17as2	0	0.1	-	-
T17as3	0	0.3	-	-
T17as4	0	0.6	glx	(glx,leu/ile,pro,gly,arg)
T17as5	0.95		ser	(ser,arg)(ala,gly,weak)

The sequence tentatively deduced from this data is:



15. T17b his,arg,asx,thr_2,gly,val,met,leu

T17b has no amides. A single,acidic chymotryptic sub-peptide was isolated, corresponding to his(asx,pro,thr,leu/val,gly).

The sequence of T17b is:

his-leu/val-asp-thr-gly-met-thr-leu/val-arg

16. T17c his-glu-thr-pro-leu-val-lys

17. T18a leu-gly-ala-arg

18. T18b gly-ser-arg

19. T18c asn-arg

Low yields of histidine in the amino-acid analysis were noted with both T17b and T17c in which histidine is N-terminal. A similar phenomenon was observed by Meadway (1969b) with a peptide with N-terminal histidine.

The following peptides from Table 11 are also of particular interest.

20. T2a. The amino acid composition of T2a suggests that it was impure, although the N-terminal determination and six cycles of

sequential degradation were satisfactory. A similar peptide was isolated in very poor yield in the fourth digest. Cysteine (as cysteic acid) was definitely present. This is the only tryptic peptide isolated which contains cysteine.

21. T6d and T7 may be peptides that were further digested under the different conditions prevailing in other digests.

22. T12a was isolated only once, in poor yield and a single, unsatisfactory amino-acid analysis indicated that it was impure. The presence of tryptophan, lysine and histidine suggests that it may be the C-terminal tryptic peptide, in an impure state.

23. T16a has been isolated and purified from every tryptic digest but has given equivocal results with respect to analysis and sub-digestion. The most probable composition is:

$$gly,ala,val,leu_2,thr_2,asx_2,glu_2,(phe),met,his,arg,lys.$$

Phenylalanine was always in low yield relative to the other amino-acids. The following peptides have been isolated after chymotryptic digestion.

Peptide	m	m'	N-terminus	Composition
T16ac1	-0.4	0.07	glx	(phe,glx,val/leu,ala)
T16ac2		0.1	(asx)	(asx,glx,arg)(gly,ala)
T16ac3	0.6		(val/leu)	(met,asx,thr,leu/val,gly, his,lys)

A partial and tentative sequence is:

glu-leu-val-(gly,ala,leu,thr_2,asx,met,his,(phe),lys)-asp-glu-arg

24. T19a is free serine, isolated in one tryptic digest with a yield of 1%.

25. T19C was the only ninhydrin-negative peptide isolated in tryptic digests. No N-terminus was apparent, despite three attempts with the two isolates of this peptide. The yield was very low and the composition as determined by electrophoresis at pH 2.0 was glx, and leu/ile, with traces of gly and ala also present. Its mobility at pH 6.5 was 0.0 and Rx in BAWP was 0.8.

Table 12 lists some peptides isolated in very low yield and successfully analysed.

Carbohydrate

Carbohydrate material was detected with O-dianisidine in one major tryptic digest. The carbohydrate material appeared to be associated with, but not covalently bound to, free amino-acids, as they could be separated by electrophoresis at pH 2.0. The carbohydrate material chromatographed in the same position as D-glucose on BAWP, gave thessame yellow-brown colour as glucose with O-dianisidine and was estimated, from the relative strengths of the spots, to represent about 0.03 µmol of carbohydrate in toto.

Peptide Yields

Amino acid analysis of samples, taken from the third and fifth tryptic digests, indicated that approximately 80% and 70% respectively

of the β-lactamase digested was applied to the primary fractionation column, and that the insoluble material made up the remainder. The primary fractions were not analysed to check the recoveries from the first stage, but other evidence (see Chapter IV and Chapter VIII) suggests that both cation-exchange chromatography and gel filtration give good recoveries of peptides.

All of the peptides isolated required further purification by paper electrophoresis and chromatography, for which the average peptide recovery per stage is about 40-50%. Two, three or even four stages were often required so that peptides present in good yield in the digest were unlikely to be recovered in yields greater than 10-20%.

Ambler (1963b) recovered tryptic peptides from a 82-residue cytochrome in yields of 10-45%. Ambler and Brown (1967) recovered tryptic peptides from a 128-residue azurin in yields of 5-30% and ascribed the lower yields in this case to the increased size of the protein. The peptide yields in the present work would be expected to be lower again, although not generally as low as was actually found. Schroeder et al. (1963) initially purified tryptic peptides from γ-haemoglobin (146 residues) by cation-exchange chromatography and noted yields of 40-95% for most peptides but further purification by paper electrophoresis or anion-exchange chromatography was necessary in most cases, and occasioned further losses.

The "multiple forms" of some peptides and the discovery of some in more than one primary fraction aggravated the problem of low peptide yields. In some cases, commencing analytical work on peptides which were subsequently shown to be impure was responsible

for equivocal results and wasted material.

The third digest, in which the protein was oxidised and digested in urea gave generally the best yields, particularly of some of the acidic peptides. Some of the basic peptides (e.g. T16a, T17b, T17c) were in best yield in the second digest, in which no denaturation was used. The fourth digest gave the poorest yields but this may have been due to the more extensive paper electrophoresis necessary to purify the peptides.

Cation-exchange chromatography was the better primary fractionation procedure. Although initially involving more work than the Sephadex method, it gave rise to several relatively simple primary fractions rather than a few complex ones. If a longer column had been used, the primary fractionation might have been even more effective. One disadvantage of this method was that the peptides in a primary fraction were often of similar size and charge, so that it was difficult to decide upon a subsequent purification method. There was no evidence for the irreversible binding of peptides to the cation-exchange resin.

Specificity

There was no evidence from any of the major peptides for cleavage other than at the C-terminus of lysine or arginine, but the peptides have not all been entirely sequenced, so that this conclusion may be premature. There is certainly evidence for peptide bonds of varying degrees of susceptibility to trypsin; cf. T6a with T15a and T11a with T15b. The latter case is an example of an adjacent polar residue, in this case glutamic acid, inhibiting tryptic cleavage. This phenomenon has been frequently observed (Hill, 1965).

Table 13. The Extent of the Tryptic Peptides

	Whole Protein	Tryptic Peptides
gly	14	13
ala	16	14
val	9	6
leu	19	15
ile	9	7
ser	11	9
thr	14	10
asp	17	16
glu	19	15
phe	4	(5)
tyr	3	1
trp	3	1
cys	1	
met	6	6
pro	7	9
lys	9	8
his	4	3
arg	11	12
	176	150

The Extent of the Tryptic Peptides

Table 13 compares the numbers of amino-acids accounted for by the peptides listed in Table 9, plus T16a, with the amino-acid composition of β-lactamase in mols/mol calculated in Chapter II. The C-terminal region of the molecule and the sequence around the cysteine residue are probably not accounted for in the "tryptic peptides" total.

In general the comparison suggests that the "tryptic peptides" comprise most but not all of the β-lactamase molecule. However, some of the amino-acids are present in greater numbers than in the amino-acid composition, and inclusion of the poorly-characterised tryptic peptides would increase this imbalance, although some regions of sequence would then almost certainly be represented more than once.

Four possibilities must be considered:

(i) The protein was impure.

(ii) The amino-acid composition is inaccurate.

(iii) The protein has a higher molecular weight than has been calculated.

(iv) The tryptic peptides are equivocal.

The data presented in Chapters II and III suggest that the first possibility is unlikely to make a major contribution to this phenomenon, as the peptide yields from a small contaminant would be vanishingly small.

The discussion of amino-acid analysis in Chapters II and IV has emphasised that considerable errors are possible here.

The third possibility is worthy of further consideration here, as the analytical data presented in Chapter II gave a considerable

range of estimates of molecular weight, and results from the amino-acid sequence data were used in order to arrive at a probable mean value. In view of the extensive production and purification of tryptic peptides, it is probable that the peptides listed represent 90% of a 180-residue protein, rather than about 65% of a 230-residue protein.

The final possibility probably contributes to the discrepancy between the composition of the tryptic peptides and of the whole protein. In those peptides which have been sequenced entirely, there are several examples of different peptides which are derived from the same region of sequence. Peptides such as T6d and T7 can probably be hydrolysed further.

An Experiment With ETPA

The reversible reaction of the ϵ-amino groups in lysine residues with the anhydrides of dicarboxylic acids so as to restrict tryptic cleavage to arginine residues is now well established as a technique in amino-acid sequencing (Butler and Hartley, 1972). It was felt that this technique would be useful in establishing the "-lys-leu-" and "-leu-lys-" overlaps in the β-lactamase sequence.

Recently, Riley and Perham (1970) have introduced exo-cis-3,6-endoxo-Δ^4-tetrahydrophthalic anhydride (ETPA) as a reagent which forms a lysine amide derivative which is stable at neutral pH but which can be hydrolysed under relatively mild acid conditions which are not liable to cleave peptide bonds. The half-lives of maleyl-ϵ-lysine and citraconyl-ϵ-lysine, which have been used in this role in the past, are 11 h at pH 3.5 and 20 min at pH 2.0 respectively. ETP-ϵ-lysine has a half life of 5 h at pH 3.0. In addition, ETPA does not have an activated olefinic double bond

which might lead to alkylation of cysteine residues, and the diastereo-isomers of ETP-acetyl-lysine amide are hydrolysed at the same rate, and are not electrophoretically distinguishable (Riley and Perham, 1970).

ETPA was prepared by a Diels-Alder condensation between maleic anhydride and furan (Riley and Perham, 1970). 10 g maleic anhydride (BDH Laboratory reagent recrystallised from chloroform) and 7.3 ml furan (BDH) were mixed in a round-bottomed flask and warmed gently under reflux. The maleic anhydride dissolved and a different crystalline precipitate formed. On cooling, the solid was broken up and washed with cold water. It was recrystallised from chloroform and found to have a melting-point of 113°C (115-116°C, Riley and Perham, 1970).

7 µmole of protein was dissolved in 10 ml of 0.2 M sodium borate buffer pH 8.5. 170 mg of ETPA was added in portions of about 20 mg, whilst stirring at 4°C. The pH was maintained at 8-9 with 1 M NaOH. After 45 min, the ETPA had dissolved and the preparation was freeze-dried, desalted on Sephadex G-25 in 0.1 M ammonia and again freeze-dried. Unfortunately, the assays performed before and after this procedure to determine free amino groups were below the sensitivity of the assay technique (see Habeeb, 1966, Chapter IV).

The preparation was digested with DPCC-trypsin and the digest fractionated on a column of Sephadex G-25 (superfine) in 0.1 M ammonia. Three fractions were isolated and the ETP-groups were removed from each by incubation in 0.1 M sodium citrate buffer pH 3.3 for 40 h at room temperature. Fraction E2 was subsequently shown to consist of T15b, T18b and T18c, and E3 of T18c and free

arginine.

Fraction E1 precipitated in a Sephadex G-50 column in 0.1 M ammonia and was removed with 8 M urea. It was re-run on the column in 0.1 M ammonia containing 8M urea and four fractions were isolated and precipitated with TCA. About forty peptides, some ninhydrin-negative and some recognisable as tryptic peptides, were purified from these fractions, but the yields were extremely low and the peptides were often insoluble.

A similar complexity was observed on the maleylation of a histone from chicken erythrocytes and was ascribed to steric hindrance of maleylation at adjacent lysine residues (Greenaway, 1970). Reaction of ETPA with poly-L-lysine, under the conditions described above, indicated that more than 90% of the amino groups were blocked. Thus this hypothesis, even if it could be applied to β-lactamase, is untenable.

It is possible that the ETP-groups were not entirely removed by the "unblocking" procedure. ETP-esters of serine, threonine and tyrosine may have added to the complexity of the experiment but the "unblocking" reaction should also hydrolyse these derivatives (Riley and Perham, 1970).

If none of the reactions involved went entirely to completion then a complex mixture of minor peptides would be expected. This is the most probable explanation of the results of this experiment.

CHAPTER VI.

CHYMOTRYPTIC PEPTIDES OF E.COLI β-LACTAMASE

Three chymotryptic digests were performed (Table 8, Chapter IV) but the first, small-scale experiment will not be discussed here.

The data relating to the chymotryptic peptides is summarised in Tables 14 and 15. Some of these peptides are discussed below and the sequences or partial sequences are summarised in Table 16.

Chymotryptic Peptides

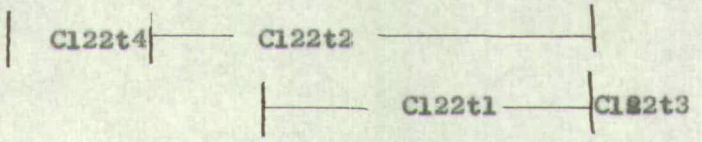
1. C122 $lys_3, asx_2, glx, ala, val_2, leu$

C122 has no amides. It was sub-digested with trypsin. The sub-peptides were qualitatively analysed.

Peptide	m	N-terminus	Composition
C122t1	-0.8	asx	(asx,glx,leu/ile,ala,lys)
C122t2	-0.2	val	(asx,glu,leu/ile,val,lys,ala)
C122t3	0.0	leu	leu/ile
C122t4	+0.9.	val	(val,lys)

C122H, C122t2 and C122t4 were tentatively identified as the tryptic peptides T6a, T15a and T18d respectively. The partial sequence is:

val-lys-val-lys-asx-(lys,asx,glx,ala)-leu



2. C123 $lys, his, arg_2, met, thr, glx_2, pro_2, val, phe$

C123 has no amides. It was sub-digested with trypsin and the sub-peptides were qualitatively analysed.

TABLE 14 CHYMOTRYPTIC PEPTIDES

Peptide	m 6.5	V/Vo G-25	Putn	Yield %	Gly	Ala	Val	Leu	Ile	Ser	Thr	Asp	Glu	Phe	Tyr	Trp	Cys	Met	Pro	Lys	His	Arg	TOT	N-t	-1	-2	-3	-4	-5	-6	-7
C11	0·5	1	S6	1-3	3·8	4·0	0·1	1·1	2·0	1·7		1·0	0·9							1·1		2·0	18	ser	—						
C121	0·2	1	S63	1	1·0	0·3	0·1	1·1		1·2		3·2	0·9						1·0	1·2			9	lys	asx	glx	—				
C122	0·2	1	S63	2		1·0	2·0	1·1				2·2	1·0							3·2			10	val	lys	val	lys	asx	—		
C123	0·2	1	S63	1	0·1	0·1	1·1	0·3			0·9		2·2	1·0				1·1	2·0	1·0	1·0	2·1	12	arg	—						
C124a	0·2	1	S63	1		1·1		1·4	1·2			2·2						0·9	1·0	1·9			9	leu	asx	(ile)	—				
C125b	0·2	1	S63	1·5	2·0		0·2	1·3	1·3	0·3	1·8	0·2	1·1						1·0	1·4	0·1		9	Hr	Hr	gly	—				
C142	-0·8	1	S63	1		2·9			0·9		2·1	3·2	2·3					2·0	2·0			1·4	16	glx	pro	—					
C242b	-0·15	2	S63	2·5	0·7	1·1		0·7	1·0	0·8		1·0	2·1									1·3	9	asx	—						
C251	-0·15	2	S63	1						0·9			2·0	2·2				1·1	1·8			2·2	10	glx	ser	phe	arg	pro	glx	—	
C261b	-0·45	2	S63	2		2·2		1·0		1·2	1·0	3·0											8	ser	asx	—					
C271	-0·75	2	S6	4	0·1	2·0			1·0	1·1	1·0	0·1					1·0	1·0					7	—							
C331a	0·75	3	S63	3	0·3	0·9		0·9		1·1			1·8									1·0	6	ser	glx	—					
C59	0·8	—	C63	2	0·2	1·0	0·1			0·8	0·1	0·2	1·9						0·2	0·2		1·0	5	ser	glx	(glx)	—				
C331b	0·75	3	S63	1·5	0·3	0·8	0·3	1·2		0·9			1·0							0·8		1·2	6	arg	(lys)	—					
C332	0·85	3	S63	1		0·2		1·0	0·2	0·2								0·3		1·0	0·3		2	lys	leu						
C34	0·4	3	S6	2	1·2	2·1		1·2		0·9				0·9				0·9				0·8	8	arg	ser	(gly)	leu	—			
C352a	0	3	S632	5		1·0		1·2		0·1	1·8												4	ala	Hr	Hr	leu				
C352c	0	3	S132	12				1·0		0·2													1	leu							
C392a	-0·55	3	S683	2	0·1		1·0	1·0		1·0		1·8	2·1		0·4								8	ser	glx	(asx)	(val)	—			
C392b	-0·55	3	S683	2						1·2		0·3	1·0	0·8				0·3					3	glu	ser	(ser)	—				
C393b	-0·55	3	S683	5				2·1	2·0	0·2			1·1										5	leu	glu	ile	ile	(leu)			
C42	0·6	4	S63	2-4	2·3	1·3	1·3								0·6							2·0	7	gly	ala	arg	—				
C45	0·0	4	S83	8						1·0	1·0			1·0				1·0					4	met	ser	Hr	phe				

TABLE 15 CHYMOTRYPTIC PEPTIDES

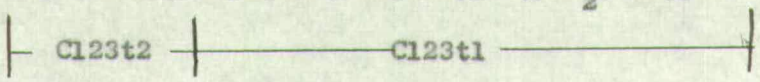
Peptide	m 6.5	V/Vo G-25	Putn	Yield %	Gly	Ala	Val	Leu	Ile	Ser	Thr	Asp	Glu	Phe	Tyr	Trp	Cys	Met	Pro	Lys	His	Arg	TOT	N-t	-1	-2	-3	-4
C124b	0·2	1	S63	–	1·9	0·2	1·0	1·2	0·2	0·3	1·3	1·1	0·3					0·1		1·0		0·2	7	lys				
C125a	0·2	1	S63	1°				1·0			0·2	2·0								1*			4	asx				
C141	-0·8	1	S63	1	0·3	1·3	0·3	0·3	0·3	1·0	1·0	2·0	2·2	0·8				0·8		1·0			10	ala				
C15	0·0	–	C6382	–	0·2	1·1	0·3	0·9	0·4	0·2	1·0	2·2	1·8					0·4			0·3	1·0	8	glx				
C216	0·0	–	C32	1	0·3			1·8			0·9	0·1	1·0						0·2		0·3		4	thr				
C311	0·0	–	C65	0·5	1·0	0·1						0·2	0·2										1	gly				
C32	0·9	3	S6	–			1·0	2·0												1*			4	lys	val	leu	–	
C33	0	–	C6383	–	* 1	1	1	1		1		1	1									1	8	ser	arg	–		
C391a	-0·55	3	S63	5	0·8	1·3	1·0	1·1				0·2											4	gly				
C393a	-0·6	3	S633	–				1·0		0·4			0·9										2	–				
C433	-0·6	–	C32	0·5			0·8	0·8		1·4	0·4	1·8	1·9			+ +			0·4	1·0	0·9		10	ser				
C46	0·2	–	C6	1	1·2	0·8	0·8	1·0	0·3	1·0	0·9	1·0	1·2						0·8	1·1			10	ser				
C70	0·0	–	C6	–	*		1	1		1	1	1	1									1	7	thr	val	glx	asx	–
C71	0·2	–	C63	3	1·1	0·4	0·9	1·0	0·1	0·4	1·0	2·3						0·1		0·2	1·0	0·2	7	leu	lys	asx	gly	–

* not quantitated.

Peptide	m'	N-terminus	Composition
C123t1	0.3	-	(met, glx,thr,leu,ile,val)
C123t2	0.6	arg	(glx,arg)

A tentative, partial sequence for C123 is:

arg-glu-arg-(lys,his,met,thr,glu,pro_2,val,phe)



3. C125b $lys,thr_2,glx,pro,gly_2,leu,ile$

The mobility of C125b (m = 0.2) suggests that it has one amide. It was sub-digested with trypsin and the sub-peptides were qualitatively analysed.

Peptide	m'	N-terminus	Composition
C125bt1	0.3	glx	(glx,leu/ile)
C125bt2	0.6	thr	(pro,thr,leu/ile,gly,lys)
C125bt3	1.0	-	(gly,lys)

C125bt1 is apparently a neutral peptide of composition (glx,leu/ile) and with glx as N-terminus. Thus C125bt1 is probably gln-(leu/ile). The data does not permit the derivation of a partial sequence beyond what is known from sequential degradation:

thr-thr-gly(lys,gln,pro,gly,ile,leu)

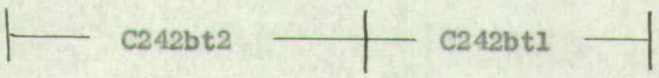
4. C242b $arg,asx,ser,glx_2,gly,ala,ile,leu$

C242b probably has one amide. It was sub-digested with trypsin.

Peptide	m'	N-terminus	Composition
C242bt1	0.15	ile	(glx,leu/ile,ala)
C242bt2	0.3	asx	(asx,glx,leu/ile,lys)

A partial and tentative sequence for C242b is:

asx-(glx,ser,gly)-arg-ile-(glu,ala)-leu



In one chymotryptic digest, peptide C242a (m = -0.25) was isolated, in very low yield. Its N-terminus and qualitative composition corresponded to that of C242bt1.

5. C251 arg_2,met,ser,glu_2,pro_2,phe_2

C251 is slightly acidic, for which there is no obvious explanation. It was sub-digested with subtilisin and the sub-peptides were qualitatively analysed.

Peptide	m	N-terminus	Composition
C251s1	-0.25	glu	(phe,glu,pro,ser,leu/ile,arg)
C251s2	-0.2	glu	(phe,glu,pro,arg)
C251s3	0	-	-
C251s4	0.05	glu,SAG	(met,phe,glu,pro,ser,leu/ile,arg)

C251s4 was obviously impure. It is not possible to elaborate on the partial sequence deduced from sequential degratation.

glu-ser-phe-arg-pro-glu-(arg,met,pro,phe)

6. C271 cys,met,thr,ser,ala_2,ile

An N-terminal residue was not detected, although Dns-$cySO_3$ is very acidic at pH 4.4 and could be lost in the electrophoresis very easily. The peptide was only isolated on one occasion.

Sequential degradation was unsatisfactory. The results suggest that some residues were not completely removed, resulting in "raggedness".

C271 was sub-digested with thermolysin and the sub-peptides were qualitatively analysed.

Peptide	m'	N-terminus	Composition
C271h1	0.3	ile	(met,thr,leu/ile,ala)
C271h2	0.4	SAG	(ala)

No other peptides were detected, although the chlorination method for ninhydrin-negative peptides was not used. A small peptide containing cysteic acid would be very acidic, and could perhaps be lost.

A very tentative sequence is:

cys-ser-ala-ile(thr,ala)met

C271h1 — C271h2

7. C331a arg,ser,glx_2,ala,leu

The mobility of this peptide suggests that it has two glutamine residues and this may explain the failure of the sequential degradation.

ser-gln-(arg,gln,ala)-leu

8. C59 arg,ser,glx_2,ala

C59 is possibly homologous with C331a. On this basis of previously-observed chymotryptic cleavages (Hill, 1965), glutamine is most likely to be the C-terminal residue in C59.

Both C59 and C331a were isolated in comparable yields in the third digest.

9. C34 arg,ser,pro,gly,ala_2,leu,phe

An attempted papain sub-digest was unsuccessful, the unreacted peptide and a very faint neutral peptide being recovered. A tentative sequence can be derived if it is assumed that a "-leu-pro-" sequence is present. At least one case of chymotryptic cleavage under similar circumstances has been reported (Léonis et al., 1959)

arg-ser-gly-leu-pro-ala-ala-phe

10. C392a asx_2,ser,glx_2,val,leu,tyr

C392a has two amides. The yield of tyrosine in the amino-acid analysis was low, perhaps due to incomplete evacuation of the sample tube prior to acid hydrolysis. An unusual peak on the amino-acid analyser trace was possibly "tyr-X" (Sanger and Thompson, 1963). C392a was not digested further by chymotrypsin. The partial sequence is:

ser-glx-asx-val-(glx,asx,leu,tyr)

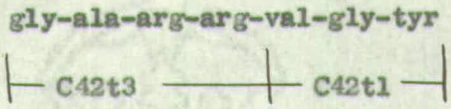
11. C42 gly_2, ala, val,tyr,arg_2

The amino-acid analysis for this peptide was rather poor, but the mobility is compatible with a heptapeptide with two positive

charges at pH 6.5. It was sub-digested with trypsin.

Peptide	m	N-terminus	Composition
C42t1	0.1	val	(tyr,leu/val,gly)
C42t2	0.6	weak, probably C42	
C42t3	0.9	gly	(ala,gly,arg)

A tentative sequence based on this data is:



Of the peptides listed in table 15, some are of particular interest.

12. C216 thr,glx,leu_2

This is a neutral peptide, so probably contains glutamine rather than glutamic acid. The N-terminus was threonine.

The N-termini observed during four stages of sequential degradation were:

thr^{+}/glu^{+}

glx^{++}

glx^{++}/leu^{++}

leu^{++}

The sequence is probably:

thr-glu-leu-leu

13.

The composition of C352b can be written as ($thr_{2.5}$,$ala_{2.5}$, leu,$phe_{2.5}$) or as (thr,ala,$leu_{0.4}$,phe). In view of the fact that C352c was free leucine in high yield, it is probable that C352b consisted of the peptide thr-ala-phe and free leucine as a contaminant.

14. C70 (asx,thr,ser,glx,leu,val,arg)

Despite three attempts, only a semi-quantitative analysis of this peptide was achieved. It probably has one amide. Attempts to sub-digest this peptide with trypsin and subtilisin were unsuccessful.

Discussion

The results of two chymotryptic digests of β-lactamase have been presented. The peptide yields were generally lower than for the tryptic peptides. This phenomenon is due to the broader specificity of chymotrypsin and is generally observed during sequence analysis (Ambler, 1963b; Ambler and Brown, 1967). In the second experiment, in which digestion was for 4 h, the peptides were initially fractionated by gel filtration. In the third experiment, ion-exchange chromatography, which was most effective in the initial fractionation of tryptic peptides, was used, but the digestion had continued overnight and the peptide mixture was much more complex. Thus in both cases extensive purification by paper electrophoresis was necessary and this factor certainly contributed to the low yields.

The total amino-acid composition of all the chymotryptic

peptides described is considerably higher than that of the β-lactamase molecule but in view of the broad specificity of chymotrypsin and the possibility of phenomena such as deamidation and the overlap of primary fractions (discussed in Chapter V), this is not surprising. Most of the peptides isolated in the second digest were also isolated in the third. In both digests, but especially in the third, some peptides were purified out of existence and some were isolated impure, in very low yield; these peptides have not been reported here. Thus there may be some regions of the β-lactamase amino acid sequence which are not represented in the chymotryptic peptides. No attempt was made in either case to fractionate the insoluble portion of the digest.

The paucity of data relating to the C-termini of the chymotryptic peptides precludes any significant review of the specificity of chymotrypsin but no hitherto unobserved examples of chymotryptic cleavages need be postulated.

Despite the fact that little is known about its sequence, C271 is a small, and almost certainly pure, peptide containing cysteine. It is not possible to reconcile what is known about C271 with what is known about T2a, which also contains cysteine, but the known impurity of the latter may account for this.

The isolation of C271 prompted an attempt to isolate a cysteine containing sequence from β-lactamase digested with subtilisin by the "performic acid diagonal" technique (Brown and Hartley, 1966). The experimental details are given in Appendix III. A single, large, impure, cysteine-containing peptide was isolated in low yield. No further characterisation was possible. The experiment gave further evidence that there is a single cysteine residue in E.coli β-lactamase.

Table 16. Partial Sequences of Chymotryptic Peptides

C11	ser(lys,arg_2,asx,ser,glx,gly_4,ala_4,ile_2,leu)
C121	lys-asx-glx(ser,asx_2,pro,ala,leu)
C122	val-lys-val-lys-asx-(lys,asx,glx,ala)-leu
C123	arg-glu-arg-(lys,his,met,thr,glu,pro_2,val,phe)
C124a	leu-asx-ile-(lys_2,asx,met,pro,ala)
C125b	thr-thr-gly-(lys,glx,pro,gly,ile,leu)
C142	glx-pro-(ile,arg,asx_3,met_2,thr_2,glx,pro,ala_3)
C242b	asx-(glx,ser,gly)-arg-ile-(glu,ala)-leu
C251	glu-ser-phe-arg-pro-glu-(arg,met,pro,phe)
C261b	ser-asx-(asx_2,thr,ala_2,leu)
C271	cys-ser-ala-ile-(thr,ala)-met
C331a	ser-gln-(arg,gln,ala)-leu
C59	ser-gln-(arg,ala)-gln
C331b	arg-lys-(ser,gln,ala)-leu
C332	lys-leu
C34	arg-ser-gly-leu-pro-ala-ala-phe
C352a	ala-thr-thr-leu
C352c	leu
C392a	ser-glx-asx-val-(glx,asx,leu,tyr)
C392b	glu-ser-phe
C393b	leu-glu-ile-ile-leu
C42	gly-ala-arg-arg-val-gly-tyr
C45	met-ser-thr-phe
C124b	lys-(asn,thr,gly_2,val,leu)
C125a	asn-(asn,lys,leu)
C141	ala(lys,asx_2,met,thr,ser,glx_2,phe)

Table 16. - Continued.

C15	glx(ala,thr,asx_2,glx,arg,leu)
C216	thr-glu-leu-leu
C311	gly
C32	lys-val-leu-leu
C33	ser-arg-(asx,glx,gly,ala,val,leu)
C391a	gly-val-ala-leu
C393a	glu-leu
C46	ser-(gly,ala,val,thr,asn,glu,pro,lys,leu)
C70	thr-val-glx-asx-(arg,ser,leu)
C71	leu-his-asx-gly-(asx,thr,val)

CHAPTER VII.

PEPTIC PEPTIDES OF E.COLI β-LACTAMASE

A preliminary experiment, at pH 2.0 and with an enzyme/substrate ratio of 1/40, indicated that after 4 h peptic digestion was relatively complete. Digestion in 2 M urea was not as effective.

The conditions used for the single, large-scale peptic digest have been summarised in Table 8 (Chapter IV). 9.5 μmol β-lactamase was preincubated at pH 2.0 and digested for 4 h at 37°C with 5 mg of pepsin. After freeze-drying, the residue was extracted with 0.1 M-ammonia and the extract was fractionated on Sephadex G-25 in 0.1 M-ammonia. The peptide map of this fractionation is shown in Figure 20. A portion (1/25) of each of the four primary fractions was analysed for amino-acids.

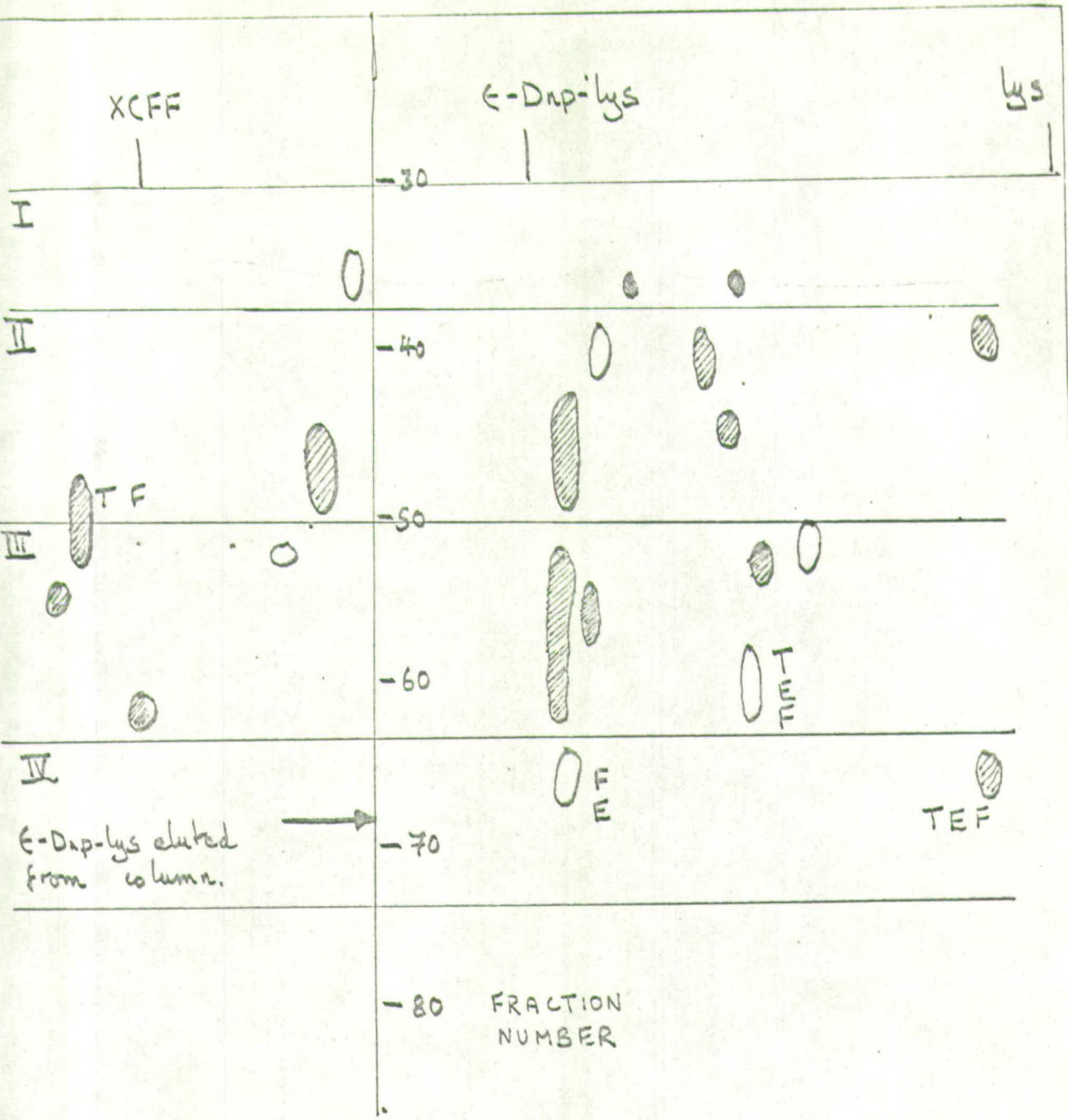
The insoluble residue of the digest, the so-called "peptic core" was dissolved in 2.5 ml 0.1 M-ammonium acetate pH 4.0, 8 M in urea, and a portion was analysed for amino-acids. Attempts to fractionate the remainder by cation-exchange chromatography on CM-cellulose in the same buffer were unsuccessful. After desalting and freeze-drying, the residue was found to be insoluble in 50% formic acid and in equal volumes of 5% formic acid and ethanol, and so gel filtration was impractical.

The peptic peptides of E.coli β-lactamase are summarised in Tables 17 and 18. Peptides P271, P272, P341, P342 and P343 were accidentally lost.

Peptic Peptides

1. P244b $asx_3, glx_3, ala, ile, arg_2$

P244b probably has two amides. It was sub-digested with



FRACTIONATION OF PEPTIC PEPTIDES BY SEPHADEX-G-25 GEL FILTRATION

PEPTIDE MAP SHOWING FOUR MAIN FRACTIONS

T = tyrosine/histidine peptide E = tryptophan peptide

F = fluorescent peptide

FIGURE 20

TABLE 17 PEPTIC PEPTIDES

Peptide	m 6.5	V/Vo G-25	Putn	Yield %	Gly	Ala	Val	Leu	Ile	Ser	Thr	Asp	Glu	Phe	Tyr	Trp	Cys	Met	Pro	Lys	His	Arg	TOT	N-t	-1	-2	-3	-4	-5	-6
P130	0.05	1	S63	5	1.9	1.8	2.3	1.0	0.8	0.3	0.3	1.9	2.3	0.8	0.7					2.3		0.9	17	LVIF	(tyr)	gly	—			
P140	0.2	1	S6	—																										
P211	-0.8	2	S633	—																										
P212	-0.8	2	S633	0.2	0.3	0.2	0.3	1.0	0.1	0.3	0.8	1.9	1.3										5	asx						
P222a	-0.55	2	S633	0.7	0.2	1.2	0.2	0.3	0.8	0.3	1.3	3.2	1.9						0.8				9	—						
P222c	-0.55	2	S683																											
P224	-0.5	2	S683	1	0.4	2.1				0.3		0.4	2.8					1.0					6	glx	glx	ala	ala	—		
P225	-0.5	2	S683	2	0.1	0.5		1.2	1.0	0.1	0.1	1.0	3.2						0.9			0.9	8	ile	arg	glx	(glx)	—		
P231a	-0.37	2	S633	3	0.4	1.9		0.3		1.1	1.0	3.2	0.3				0.1						7	ala	asx	asx	thr	—		
P231c	-0.37	2	S633																											
P242	-0.25	2	S63	3	1.0	0.2	0.1	1.9	0.9	1.5	0.1	2.1	1.2							1.0			10	asx	leu	asx	gly	—		
P244a	-0.25	2	S633	4	1.0	1.0	0.9	0.9		0.8	0.2	1.1	2.2							0.2		0.8	8	ser	arg	val	asx	ala	—	
P244b	-0.25	2	S6383	2		0.9			1.0			1.9	3.0									2.3	9	asx	—					
P245	-0.25	2	S633																											
P261	0.05	2	S63	3	0.4	1.0	0.3	1.1	1.0	0.8	0.3	1.1	1.9						0.2	0.4		0.9	8	ala	ser	arg	glx	—		
P262	0.05	2	S63	2	0.5	1.4	2.1	1.1				2.1	1.3							3.0			10	val	lys	val	lys	—		
P282	0.5	2	S63	1	1.5	0.9	0.4	1.0	1.0	1.2	0.3	2.0	1.7		0.9					1.1	0.9	2.1	14	tyr						
P321	0.0	3	S63	1	0.3	0.3	0.1	1.0	0.1	0.6	0.2	0.3	0.2										1	leu	—					
P322	0.0	3	S63	1	1.0	0.2			0.6	1.0	2.0		1.0		0.8			0.1	0.1				7	ile	tyr	thr	gly	glx	—	
P323a	0.0	3	S633			2		2		1	1												6	ser	ala/thr	leu	—			
P323b	0.0	3	S633	4	0.2	2.9	0.2	1.4	0.4	0.5	2.3	0.4		0.3				1.1	1.0				9	pro	ala	ala	—			
P325	0.0	3	S63	4	0.1	2.0	0.1	0.1	0.4	1.2	1.7	0.8	0.1	0.8				1.3	0.9				9	ser	—					
P331b	0.33	3	S63																											
P332	0.33	3	S63	12	0.1	0.9		1.0		1.0		0.1	2.2							0.1		1.0	6	ala	ser	arg	glx	glx	leu	
P333	0.33	3	S63	3	0.1		0.1	0.2		0.2		0.1	2.2	2.0					0.9			2.0	7	phe	arg	pro	glx	(glx)	—	
P350	0.72	3	S63																											
P411	0.03	4	S63											1									1	phe	—					
P442	0.03	4	S63	3	1.0	1.3	0.3									++			1.1				4	ala	pro	gly	—			
P420	0.75	4	S63	7	0.2				1.0							++				1.0	1.0		4	ile	—					

trypsin and the sub-peptides were quantitatively analysed.

Peptide	m	N-terminus	Composition
P244bt1	-0.55	asx	asx,glx,arg
P244bt2	-0.47	glx	glx_2,ala,ile
P244bt3	0	-	-
P244bt4	1.0	asx	asx,arg

It is impossible to put P244bt1 and P244bt4 in the correct order without more data. Consideration of peptide X6 (Chapter VIII) permits the deduction of the sequence.

asp-glu-arg-asn-arg-glx-(glx,ile,ala)

P244bt1 — P244bt2

P244bt4

2. P261 ala-ser-arg-glx-(glx,asx,ile,leu)

P332 ala-ser-arg-gln-gln-leu

P261 and P332 are clearly homologous and hence the sequence of P261 is:

ala-ser-arg-gln-gln-leu-(asp,ile)

The sequence of P332 was confirmed by tryptic sub-digestion.

ala-ser-arg-gln-gln-leu

P332t3 | P332t1

3. P322 thr_2,ser,gln,gly,ile,tyr

Isoleucine is present in very low yield but the ile-tyr bond might be expected to be relatively resistant to acid hydrolysis (Harfenist, 1953).

4. P321 and P411 are free leucine and phenylalanine respectively, both somewhat impure. They may represent peptic cleavage at both sides of these residues.

5. P420 ile,trp,lys,his

Digestion of P420 with various amounts of CPA released free lysine and free histidine, identified by electrophoresis at pH 2.0. Since isoleucine is N-terminal, the partial sequence of P420 is:

ile-trp-(lys,his)

Discussion

The soluble material after digestion amounted to approximately 60% of the total β-lactamase digested: the insoluble material accounted for the rest. The four primary fractions contained approximately 3%, 40%, 15% and 2% of the starting material, respectively. Thus the soluble material was entirely recovered from Sephadex G-25 gel filtration.

P420 was by far the largest component of the fourth primary fraction. Analysis of that fraction indicated that the yield of P420 prior to paper electrophoresis was about 37%. After two electrophoretic purifications, an overall yield of 8% was observed. This corresponds to a recovery of 45% per electrophoretic stage. Similar values have been observed in cases of peptides analysed, re-purified and re-analysed. This effect, coupled with the broad specificity of pepsin, was responsible for the generally low peptide yields. The imperfect fractionation by Sephadex G-25 was not observed in this experiment which may partly explain the fact that the peptide yields are generally greater and more

consistent than for the chymotryptic peptides (Chapter VI). The reported yields of peptic peptides from P.fluorescens cytochrome-C551 (Ambler, 1963b) were generally comparable with the results reported here.

About 45 peptides were observed during purification, of which about 30 were obtained pure. About 20 peptides were obtaind in sufficient quantity for quantitative structural experiments. The peptides reported here account quantitatively for about 60% of the β-lactamase molecule. Some peptide fractions were accidentally lost, and may have accounted for about 10% of the sequence.

Two discrete tryptophan-containing peptides were isolated. A third was lost with fraction 34. A cysteine-containing peptide was not isolated. There was no evidence for ninhydrin-negative peptides or for carbohydrate-containing material.

As far as can be judged from the partial sequences, the observed specificity of pepsin was as expected (Hill, 1965).

Table 18. Partial Sequences of Peptic Peptides

P212	asx-(asx,glx,thr,leu)
P224	glx-glx-ala-ala-(glx,met)
P225	ile-arg-glx-glx-(asx,glx,pro,leu)
P231a	ala-asx-asx-thr-(ser,asx,ala)
P242	asx-leu-asx-gly-(ser_2,glx,ile,leu,lys)
P244a	ser-arg-val-asx-ala-(glx_2,gly,leu)
P244b	asp-glu-arg-asn-arg-glx-(glx,ile,ala)
P261	ala-ser-arg-gln-gln-leu-(asp,ile)
P322	ile-tyr-thr-gly-gln-(thr,ser)
P323a	ser-ala/thr-leu-(ala,ala/thr)-leu
P323b	pro-ala-ala-(thr_2,ala,met,leu)
P332	ala-ser-arg-gln-gln-leu
P333	phe-arg-pro-glx-glx-arg-phe
P412	ala-pro-gly-trp
P420	ile-trp-(lys,his)

CHAPTER VIII.

CYANOGEN BROMIDE PEPTIDES OF *E.COLI* β-LACTAMASE

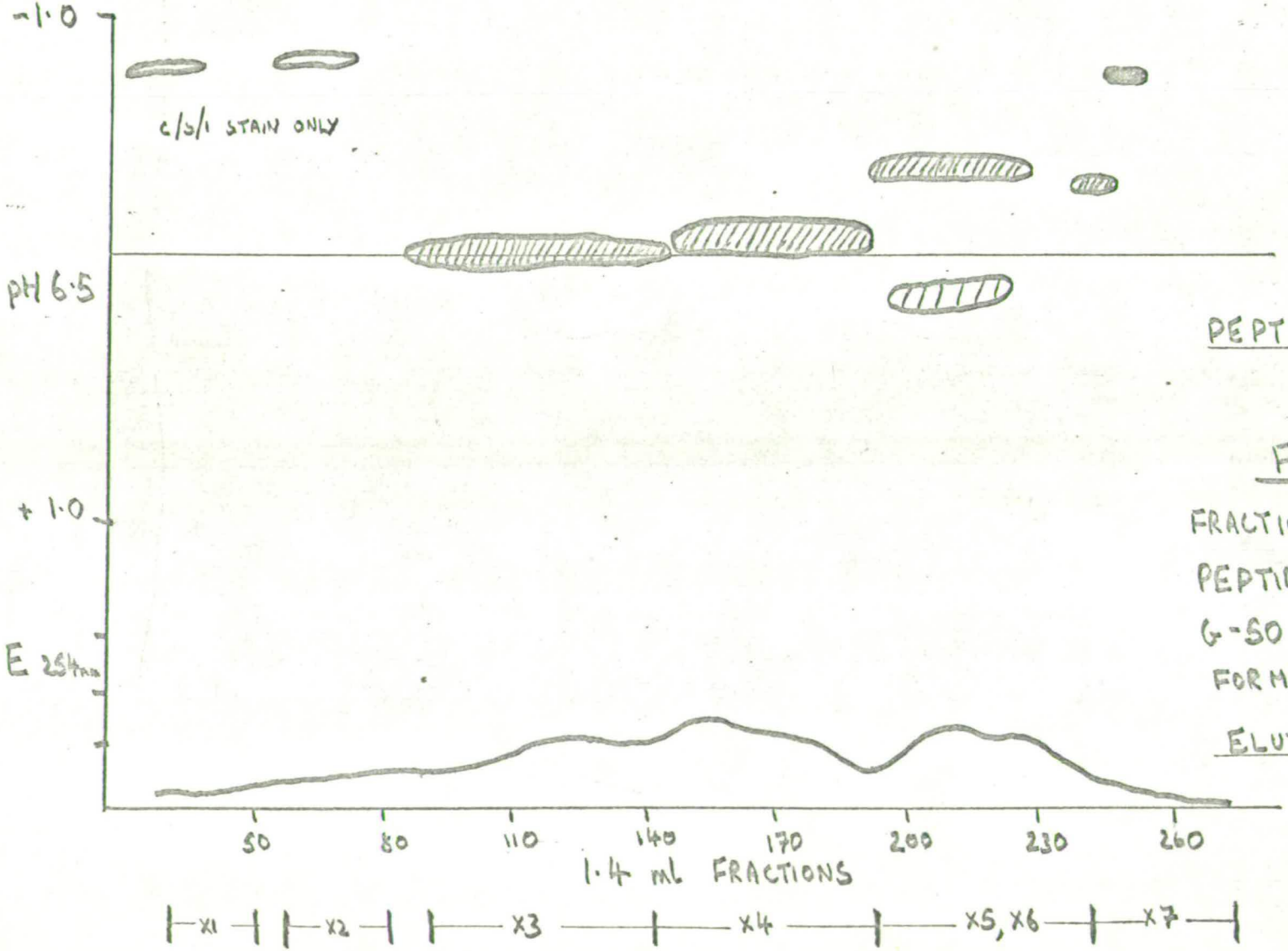
The experimental details that relate to the cleavage of β-lactamase with cyanogen bromide have been discussed in Chapter IV, and summarised in Table 8. The results presented here relate mainly to the peptides isolated in the first, second and fourth experiments. These peptides are designated 1X, 2X and 4X respectively.

Fractionation of Peptides

The elution profile (extinction at 254 nm) and the peptide map of the fractionation of the fourth CNBr experiment by gel filtration on Sephadex G-50 is shown in Figure 21. The rechromatography of fractions 4X3 and 4X4 on the same column is shown in Figure 22; prior to this experiment, fraction X4 had been considered to consist of a single peptide.

Fractions X5 and X6 were not well separated in this experiment. They were pooled together, concentrated by rotary evaporation and partially separated by chromatography and rechromatography on Sephadex G-25 in 50% formic acid. Paper chromatography in BAWP indicated that the peptides were not pure and this technique was used to effect a final purification. In the second experiment, 2X5 and 2X6 were partially separated by the initial Sephadex G-50 stage and paper chromatography in BAWP was used as the final stage. The recoveries of these peptides from the paper were rather poor.

Some of the peptides in fraction X7 gave clear spots when stained with isatin but weak spots with ninhydrin. The very basic component of X7 was not detected in the earlier experiments but



PEPTIDE MAP

FIGURE 21

FRACTIONATION OF CNBR PEPTIDES ON SEPHADEX G-50 (90 x 1 cm ; 50% FORMIC ACID)

ELUTION PROFILE

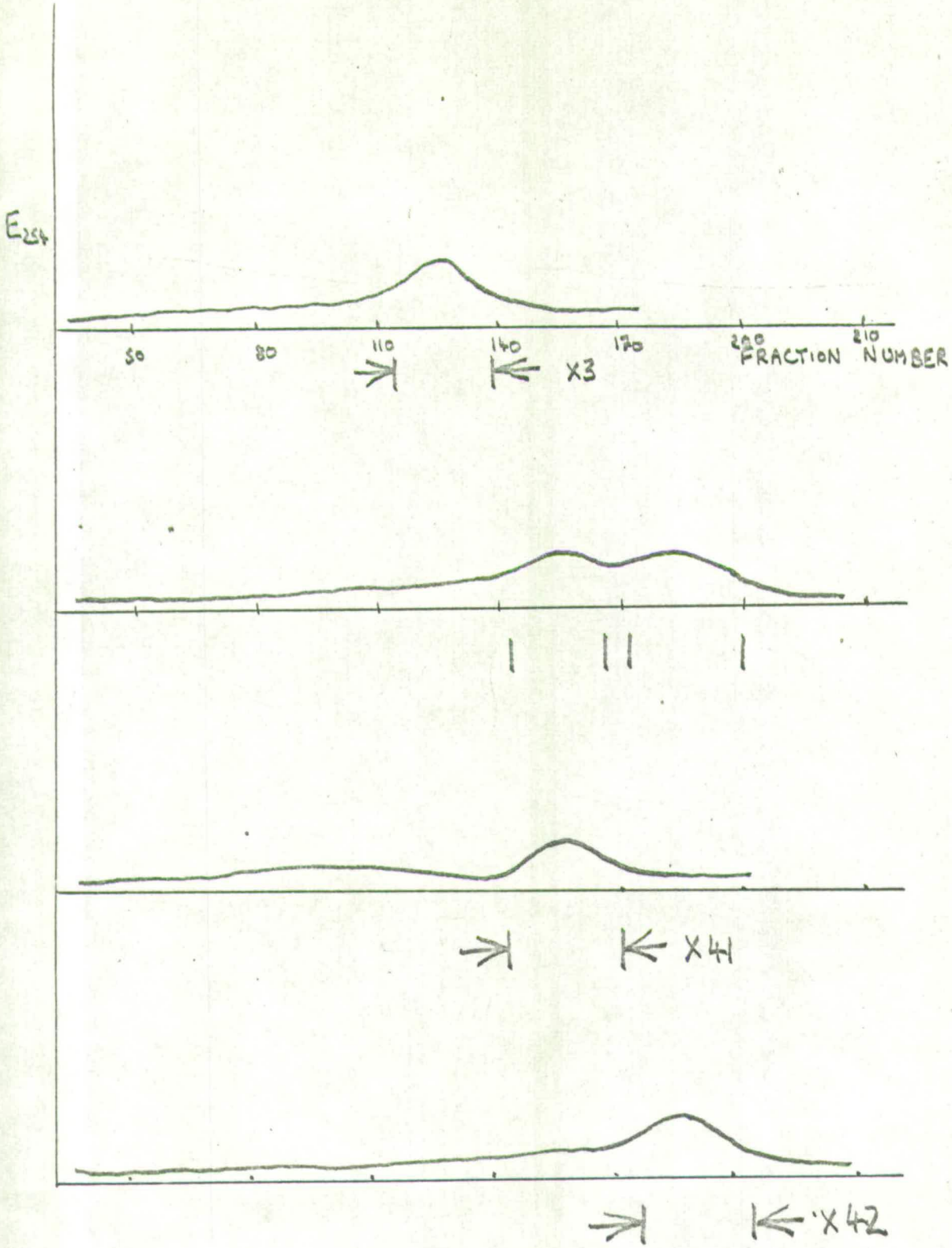


FIGURE 22 RECHROMATOGRAPHY OF CNBR PEPTIDES

could easily have been lost at the edge of the paper. The X7 components were fractionated satisfactorily by paper electrophoresis.

Fractions X1 and X2 reacted only slightly with the ninhydrin reagent but gave strong spots with the chlorine/starch/iodine reagent. The extinction at 254 nm in this region of the column effluent was very low. X1 and X2 were considered to be traces of unreacted protein or of large peptides corresponding to two or more of the other CNBr peptides.

Fractions X5, X6 and the X7 components were considered to be pure. The fact that gel filtration of X5 and X6 did not purify them suggested that X41 and X42, and, to a lesser extent X3, were not very pure. Paper electrophoresis and chromatography were not suitable for the further purification of these peptides as they simply smeared at the origin, and an attempt at cation-exchange chromatography in 8 M urea was not successful.

Fraction X7

The peptides in fraction X7 were purified by paper electrophoresis at pH 6.5 and pH 3.5.

Peptide	m	m'	N-terminus	Composition
X711	0	+0.25	pro	pro,ala_2,hsr
X712	0	0.3	(arg)	(hsr/hsl)
X72	0.1		-	-
X73	0.6		pro	pro,ala_2,hsr
X74	1.05		(arg)	hsr

X712 and X74 both gave tentative 'arg' N-termini. A sample of homoserine reacted with DNS-Cl and hydrolysed gave a similar result.

These peptides represent the homoserine and homoserine lactone forms of (pro,ala_2,hsr) and (hsr). The lactone forms predominated. No attempt was made to convert the lactone forms to the homoserine forms, except immediately before amino-acid analysis. There was no evidence for any interconversion during electrophoresis. The total yield of both forms of each peptide was of the order of 10%.

The sequence of X73 was found to be:

pro-ala-ala-hsr/hsl

Peptide X6

m	:	+0.03
R_X	:	0.4
Composition	:	trp,lys,his,arg_2,asx_2,ser,glx_3,gly,ala_2,ile_2,leu
N-terminus	:	asx
Yield	:	5-10%

X6 contained 0.8 mol tryptophan/mol peptide (by analysis), as calculated from the extinction at 280 nm (Beavan and Holiday, 1952). It did not contain homoserine or homoserine lactone and therefore is probably the C-terminal CNBr peptide.

The N-terminal sequence of X6 was determined by the "dansyl-Edman" method:

asx-glx-arg-asx-

This experiment was repeated using SPITC:

asx-glx-arg-asx-arg-glx-glx-

X6 was digested with CPA and released the following amino-acids: his, 1.0; lys, 0.7; trp, 0.5. The level of amino-acids in the

control was 0.2. No homoserine or homoserine lactone was released.

X6 was sub-digested with trypsin in two separate experiments. The compositions of the sub-peptides are qualitative, except for X6t1.

Peptide	m	m'	N-terminus	Composition
X6t1	-0.5		asx	asx,glx,arg
X6t21	0	0.27	glx	(glx,asx,ser,leu/ile,ala,gly,lys)
X6t22	0	0.32	asx	(asx,arg)
X6t3	0.1		asx	-
X6t41	0.85	0.8	(asx)	(asx,arg)
X6t42	0.9	0.9	(arg)	-
X6t5	1.0		(lys)	

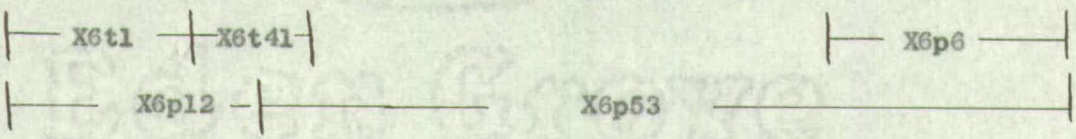
The tryptic sub-peptides confirm the N-terminal sequence of X6. X6t22 and X6t41 are probably asp-arg and asn-arg respectively. X6t21 probably represents the C-terminal tryptic sub-peptide. X6t42 and X6t5, are difficult to explain.

X6 was also sub-digested with pepsin. The sub-digest was complex but several weak peptides were lost during the purification. X6p6 was pure and was qualitatively analysed, except for tryptophan, which was detected with Ehrlich's reagent. X6p12 and X6p22 were impure and the analyses are semi-quantitative. The remaining peptides were analysed qualitatively.

Peptide	m'	N-terminus	Composition
X6p11	0.1	ala	(ala,asx,leu)
X6p12	0.15	asx	asx_2,glx,arg
X6p22	0.3	SGA	ser,gly
X6p4	0.3	ala	(ala,leu)
X6p53	0.4	arg	(glx,ser,leu/ile,ala,gly,lys)
X6p6	0.8	ile	ile,his,lys,(trp)

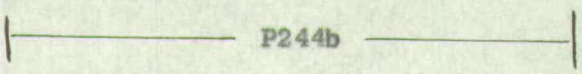
Peptide X6p6 is apparently identical with P420 (Chapter VII). The following partial sequence for X6 can be derived.

asp-glu-arg-asn-arg-glx-glx-(ile,ala_2,leu,ser,gly)-ile-trp-lys-his



The probable specificity of pepsin (Hill, 1965) and the peptides X6p22 and X6p4, together with a consideration of the C-terminal sequence of β-lactamase (Chapter III) and of the peptic peptide P244b (Chapter VII) permit the deduction of a tentative sequence for X6.

asp-glu-arg-asn-arg-glu-gln-ile-ala-ala-leu-gly-ser-ile-trp-lys-his



The N- and C-terminal regions of this sequence are reliable because of the correlation with other experiments but the region -ala-leu-gly-ser- rests on the composition of X6, on the interpretation of the CPA experiments with β-lactamase and on two poorly-characterized peptides.

Peptide X5

m : ca. -0.05 (smeared; Hsr form)

R_X : 0.25 (smeared)

Composition : $his, arg_3, asx_6, thr_3, hsr, glx_4, pro_2, gly, ala, val, ile, leu_2$

N-terminus : ala

The composition given above is for 2X5, which was judged to be very pure from the amino-acid analysis. 4X5 was less pure and analysed as:

$arg_3, asx_5, thr_2, hsr, glx_3, pro_2, gly, ala, val, ile, leu_2$

Histidine was present in very low yield and there were traces of lysine and serine. A single N-terminal alanine was observed in each case. The yield of X5 was always less than 5%.

0.05 µmol aliquotes of X5 in the homoserine form were incubated with CPA and CPB.

i) 0.01 ml CPA hsr, 0.8; leu, 0.5; hsl, 0.2.

ii) 0.1 ml CPA hsr, 0.8; leu, 0.8; hsl, 0.2; thr/asn/gln 0.8, 0.5; ala, 0.5.

The CPB sample gave a complex analysis and endoproteolytic activity is suspected. The CPA experiments suggest that the C-terminal sequence of X5 is -leu-hsr. The inability to resolve threonine, asparigine and glutamine precludes any further deductions without more evidence.

X5 was sub-digested with trypsin but was only partly soluble at pH 8.5 and the resultant peptide yields were extremely low. For a second experiment, approximately 0.4 µmol of freeze-dried X5 was

dissolved in 0.5 ml 0.3 M-ammonium carbonate pH 8.3, 6 M in urea and then diluted three-fold. 0.05 mg of DPCC-trypsin was added and the mixture was incubated for 4 h at 37°C. After freeze-drying, the digest was desalted on a small column of Sephadex G-25 in 5% formic acid and again freeze-dried. The peptide material was fractionated by paper electrophoresis at pH 6.5.

X5t23 was obtained in good yield and was reasonably pure and had the composition (ala,thr_2,leu,arg). The yield of alanine was low but an alanine N-terminus was observed. The N-terminus of X5 is alanine and examination of peptides T2d (Chapter V) and C352a (Chapter VI) suggests that the sequence of X5t23 is ala-thr-thr-leu-arg and that it represents the N-terminal sequence of X5.

X5t21 was impure but could be interpreted as (asx,glx,hsr/hsl). This cannot be reconciled with the results from the CPA experiments. Other tryptic sub-peptides were analysed qualitatively or else the quantitative analyses were uninterpretable.

A suspension of approximately 0.2 μmol of X5 in 0.5 ml 0.2 M-ammonium acetate pH 8.5, 0.005 M in $CaCl_2$ was solubilised by shaking with 0.02 ml of 0.5 mg/ml thermolysin at 37°C for 8 h. A single, basic peptide was isolated and characterised. Some neutral and acidic peptides required considerable purification and were in very low yield. X5h3 has the composition leu(ser,glx_2, ala,arg) and probably contains two glutamine residues.

It was hoped that sub-digestion of X5 would produce recognisable tryptic peptides and overlapping thermolytic peptides which would permit the deduction of a sequence for peptide X5 despite the heterogeneity of at least one isolate. This was not accomplished.

TABLE 19 THE X4 PEPTIDES

Peptide	m 6.5	V/Vo G-25	Putn	Yield	Gly	Ala	Val	Leu	Ile	Ser	Thr	Asp	Glu	Phe	Tyr	Trp	Cys	Hsr Met	Pro	Lys	His	Arg	TOT	N-t
X41					3·3	4·3	2·4	4·0	1·9	5·0	2·8	4·1	4·8	0·9	1·0			0·8	1·8	3·0	0·8	2·4		
					3	4	2	4	2	5	3	4	5	1	1			1	2	3	1	2	43	(thr)
X41 +54	0·8		63		0·3	0·1	1·0			0·2	0·9	0·3	0·2							0·4		1·2	3	thr
X41 +31	0		63							1·0													1	ser
X41 +24	-0·25		63		2	1	1	1				1	3									1	10	val
X41 +42	0·4		63		2·8	2·0		1·2	0·9	1·9	0·2	1·0	0·1						1·8	1·1		1·2	14	gly
X41 +61	0·9		63		0·9					1·0												1·0	3	gly
X42					2·4	2·4	1·9	2·6	1·0	1·6	1·4	2·0	3·0	0·4	0·4			0·8	1·3	1·8	0·4	2·1		
					2	2	2	3	1	2	1	2	3					1	1	2		2	24	—
X42+1					1·8	1·8	1·2	1·9	0·9	1·2	1·0	1·5	2·1					0·7	0·9	1·3		1·5		—

X5 is a CNBr peptide of between 20 and 25 amino-acid residues with the probable N-terminal sequence ala-thr-thr-leu-arg.

The X4 Peptides

In view of the difficulties experienced with X5, impurity, and particularly cross-contamination of X41 and X42 would not be surprising, despite the apparently effective fractionation by rechromatography on Sephadex G-50. In the second CNBr experiment, the elution profile and the peptide map did not permit a resolution of X41 and X42 and a 60-residue peptide "2X4" was isolated and studied. Thus the amino-acid compositions which are given in Table 19 are almost certainly unreliable.

The N-terminus of X41 was tentatively identified as thr/ala/pro. No interpretable results were obtained from experiments with carboxypeptidases, nor from sequential degradation.

X41 was sub-digested with trypsin in urea and a few sub-peptides were characterised (Table 19).

X41t54 is thr-val-arg. In view of the tentative N-terminus of X41 and of the sequence of T17b (Chapter V), X41t54 may represent the N-terminal sequence of X41.

X41t54 is free serine. This is particularly interesting since X41 was shown to give a positive result with the O-dianisidine reagent. If traces of free amino-acids and mono-saccharide are associated with, but not bound to, β-lactamase, it is surprising that they remain in association with a relatively large-molecular-weight fragment during gel filtration in 50% formic acid.

X41t24 gave a poor amino-acid analysis but calculation of some residues and estimation of others yielded a composition identical to that of T6b (Chapter V).

The mobility, composition and N-terminus of X41t42 are identical with those of T12b.

X41t61 is clearly identical with T18b (gly-ser-arg).

The composition and sequence of X41 are still in doubt but the probable N-terminal sequence, which overlaps a well-characterised tryptic peptide, has been isolated, together with three "internal" tryptic peptides. These four peptides total 30 residues.

The results obtained with X42 were less satisfactory. No information on the N- and C-terminal regions was obtained. Digestion with trypsin in urea was followed by gel filtration on Sephadex G-25. X42t1 was a single peak of relatively high molecular weight which was judged pure by paper electrophoresis at pH 6.5. Its composition is given in Table 19. No interpretable sub-peptides were isolated from the remainder of the sub-digest. X42t1 may consist of undigested X42.

Peptide X3

A large CNBr peptide, X3, was isolated in the first, second and fourth experiments. Attempts to purify it by cation-exchange chromatography in 8 M urea and by paper electrophoresis and chromatography were unsuccessful. Rechromatography of 4X3 was apparently effective as a purification technique (Figures 21 and 22). However, the experiences with X41 and X42 suggest that X3 was still impure.

The amino-acid composition of 4X3 is: lys, 7.7 (8); his, 2.0 (2); arg, 3.0 (3); hsr, 0.9 (1); asx, 8.7 (9); thr, 4.5 (5); ser, 3.2 (3); glx, 7.2 (7); pro, 4.2 (4); gly, 6.3 (6); ala, 8.0 (8); val, 5.5 (5); ile, 4.2 (4); leu, 7.5 (7); tyr, 2.6 (3); phe, 2.9 (3). (Trace of cysteic acid.)

TABLE 20 CNBr PEPTIDES

Peptide	m 6.5	V/Vo G-25	Purn	Yield %	Gly	Ala	Val	Leu	Ile	Ser	Thr	Asp	Glu	Phe	Tyr	Trp	Cys	Hsr Met	Pro	Lys	His	Arg	TOT	N-t
X 712	0			5-10														1·0					1	hsr
X 73	0·6			5-10		2·1												0·9	1·0				4	pro
X 6	0·03			5-7	0·9	2·0		1·3	2·4	1·0		2·0	3·0			++		0·8		1·0	0·9	1·9	17	asx
X 5	-0·05			3-5	1·0	0·8	0·9	1·8	0·9		2·8	5·6	4·0					0·9	2·0		0·9	3·0	26	ala
X 41					3·3	4·3	2·4	4·0	1·9	5·0	2·8	4·1	4·8	0·9	1·0			0·8	1·8	3·0	0·8	2·4		(thr)
X 42					2·4	2·4	1·9	2·6	1·0	1·6	1·4	2·0	3·0	0·4	0·4			0·8	1·3	1·8	0·4	2·1		
X 3					6·3	8·0	5·5	7·5	4·2	3·2	4·5	8·7	7·2	2·9	2·6		trace	0·9	4·2	7·7	2·0	3·0		—

Several attempts with each isolate of X3, using Dns-Cl according to the various procedures described in Chapters III and IV, failed to reveal the N-terminus of X3. Experiments with CPA were uninterpretable.

Sequential degradation with PITC after an initial treatment with SPITC was followed by isolating and hydrolysing the PTC-amino-acids prior to amino-acid analysis. The second and third residues were lost owing to analyser failure. The fourth residue was quite clearly arginine, pure and in fairly good yield.

Tryptic hydrolysis of X3 in urea led to the identification of the following tryptic peptides (Chapter V):

T18a, T14a, T17c, T8c, T15a.

A peptic sub-digest yielded P130 (Chapter VII) as the only recognisable peptic peptide.

The study of X3 was of use in the grouping together of a few of the tryptic peptides, but because of the possibility of X3 being impure, even these results must be viewed with caution.

Discussion

Seven CNBr peptides, containing about 190 amino-acids in all, have been isolated (Table 20). This is in reasonable agreement with the total number of amino-acids in the β-lactamase molecule, which was calculated as being 176 (Chapter II). The possible impurity of the larger CNBr peptides may mean that their true compositions are simpler and smaller in total than has been found.

Ambler and Brown (1967) discovered a 31-residue peptide, produced by the treatment of azurin with CNBr, which could not be

purified by electrophoresis but in the same study a 45-residue peptide was successfully purified using a combination of gel filtration and electrophoresis at pH 6.5. Meadway (1969b) encountered similar difficulties in purifying three large CNBr peptides (*circa* 80 residues) from *B.licheniformis* β-lactamase. Bornstein and co-workers (Bornstein and Piez, 1966; Click and Bornstein, 1970) have achieved an impressive fractionation of a wide range of CNBr peptides from the α2 chain of human skin collagen using cation-exchange chromatography and gel filtration in ammonium propionate, but similar techniques were not successful in the present study.

Hofmann (1964) observed a non-specific cleavage of trypsinogen during treatment with CNBr in 50% formic acid, presumably due to acid hydrolysis. This could be avoided at a higher pH but the CNBr reaction was not then as effective. There was no evidence for non-specific cleavage in the present work, but the possibility cannot be excluded.

Table 21. Tryptic Peptides of *E.coli* β-Lactamase

T2d/T4a	asp-thr-thr-met-pro-ala-ala-met-ala-thr-thr-leu-arg
T6a/T9a	asp-ala-gln-asp-lys
T15a	val-lys-asp-ala-gln-asp-lys
T6b	val-asp-ala-gly-glu-gln-leu-(gln,gly)-arg
T8c	val-(gln,gly,ile,leu,tyr)-asx-(asx,ser,gly,leu)-lys
T11a	leu-asp-arg-glx-glx-pro-(pro,glx,asx_2,leu/ile)-ala-(asx,glx,leu/ile)-arg
T15b	leu-asp-arg
T11b	ser-ala-LIF-ala-(trp,asx,pro,gly,ala,LIF_2)-lys
T12b	gly-leu-ile-ala-pro-(gly,asn)-ser-ala-gly-ser-lys-pro-arg
T14a	ser-gly-ala-glu-gly-arg
T14d	phe-pro-met-met-ser-thr-phe-lys
T14e	phe-pro-met-met-ser-thr-phe-lys-lys
T17a	phe-ile-glu-arg-pro-(glu,gln,leu)-ser-arg
T17b	his-leu/val-asp-thr-gly-met-thr-leu/val-arg
T17c	his-glu-thr-pro-leu-val-lys
T18a	leu-gly-ala-arg
T18b	gly-ser-arg
T18c	asn-arg
T14f	lys
T16b	arg
T2a	asx-gly-pro-cys-glx-glx-ile-phe-(asx_2,thr,glx,ala,leu)-lys
T16a	glu-leu-val-(gly,ala,leu,thr_2,asx,met,his,(phe),lys)-asp-glu-arg
T18d	val-lys

CHAPTER IX.

PARTIAL SEQUENCES IN E.COLI β-LACTAMASE

The results given in the preceding four chapters do not permit the deduction of the complete or a partial amino-acid sequence of E.coli β-lactamase. The sequences or partial sequences of several fragments, which represent a considerable proportion of the molecule, can be deduced and this task is undertaken in the present chapter. To this end, Table 21 is a convenient summary of the tryptic peptides described in Chapter V.

The fragments described here are based upon well-characterised peptides and are considered to be reliable, although some of them contain unsequenced regions. In some cases, tentative extensions of these fragments have been made. It must be strongly emphasised that any such fragment of an amino-acid sequence is based upon peptide overlaps which are equivocal until all other possibilities have been accounted for, i.e. until a partial sequence of the whole molecule has been deduced.

Fragment 1 (29 residues)

This fragment comprises the tryptic peptides T11a and T2d, which are overlapped by C142, C352a, P323b and X73 (Figure 23).

T2d was completely sequenced, and the sequences of C352a and X73 and the composition and partial sequence of P323b confirm this sequence. A tentative, partial sequence for T11a was deduced. The data relating to C142 is the quantitative amino-acid composition and the sequence of the first two residues, which are such as to overlap with T11a and T2d and to permit a little more ordering of residues within T11a. Residues 7 and 8 in fragment 1 (asx,leu)

Figure 23. Fragment 1.

T11a

leu-asp-arg-glx-glx-pro-(asx,leu,glx,pro,asx)-ala-(asx,glx,ile)-arg

T2d/T4a

asp-thr-thr-met-pro-ala-ala-met-ala-thr-thr-leu-arg

C142

glu-pro-(asx,ala,asx,glx,ile,arg,asx,thr,thr,met,pro,ala,ala,met)

C352a

ala-thr-thr-leu

P323b

pro-ala-ala-(met,ala,thr,thr,leu)

X73

pro-ala-ala-hsr

leu-asp-arg-glx-glx-pro-asx-leu-glu-pro-asx-ala-(asx,glx,ile)-arg-asp-thr-thr-met-pro-ala-ala-met-ala-thr-thr-leu-arg

are ordered "-asx-leu-" since chymotryptic cleavage after residue 8 produces C142. Residues 11 and 12 are ordered "-asx-ala-" on the basis of the N-terminal sequence of C142 and of the N-terminus and compositions of the thermolytic sub-peptides of T11a. The "-met-pro-" bond in T2d is unlikely to be cleaved by chymotrypsin, which agrees with the sequence imposed upon C142 by this overlap. The presence of C352a as an adjacent C-terminal chymotryptic peptide also supports this overlap.

The N-terminus of β-lactamase was found to be leucine (Chapter III). Fragment 1 is unlikely to represent the N-terminal sequence of β-lactamase since, if it were, there would be a twenty-residue CNBr peptide with N-terminal leucine. None of the CNBr peptides isolated had leucine as N-terminus nor did any match the amino-acid composition of the first twenty residues of fragment 1.

The CNBr peptide X5 probably overlaps the C-terminus of this fragment, as it yielded ala-thr-thr-leu-arg on tryptic sub-digestion and had N-terminal alanine. The chymotryptic peptides C331b [arg-lys-(ser,gln,ala,leu)] and C34 [arg-ser-gly-leu-pro-ala-ala-phe] are the only chymotryptic peptides with N-terminal arginine but as X5 does not contain phenylalanine, C331b is perhaps the better choice for a chymotryptic peptide overlapping fragment 1 at the C-terminus. Beyond this tentative addition, the C-terminal sequence of fragment 1 cannot be extended.

Fragment 1 may overlap at its N-terminus with fragment 2.

Fragment 2 (15 residues)

his-glu-thr-pro-leu-val-lys-val-lys-asp-ala-gln-asp-lys-leu

T17c T15a

T18d T6a/T9a

C122/P262

The tryptic peptides T17c and T6a were sequenced entirely and the N-terminal sequences and compositions of T15a, C122 and P262 confirm the sequence of fragment 2.

The C-terminal sequence of fragment 2 implies a tryptic peptide with N-terminal leucine. Only three such tryptic peptides have been isolated, one of which is presumably N-terminal, whilst the others are both accounted for by fragment 1. If most of the tryptic peptides from β-lactamase were isolated (Chapter V), it is probable that fragments 2 and 1 may overlap. The resulting sequence is 43 residues in length:

T17c-T15a-T11a-T2d

Without further evidence, either from the unequivocal assignment of all the other tryptic peptides to a place in the sequence, or from an overlapping peptide, then the overlapping of these fragments must remain tentative.

Fragment 3 (17 residues)

The discussion concerning fragments 1 and 2 suggests that peptide T18a, and not T11a nor T15b, is the N-terminal tryptic peptide. T18a and T8c are overlapped by C42, if an arginine residue (T16b) is interposed:

leu-gly-ala-arg-arg-val-gly-tyr-(gln,ile,leu)-asx-(asx,ser,gly,leu)-lys

T18a T16b T8c

C352c C42

The sequences of T18a and of C42, and the subdigestion of T8c, which placed the tyrosine residue in the N-terminal part of the peptide, are in accordance with this sequence. Free leucine

Figure 24. Fragment 2.

T18a T16b T8c

leu-gly-ala-arg arg val-(gly,tyr,gln,ile,leu)-asx-(leu,asx,gly,ser)-lys

C352c C42 P242

leu gly-ala-arg-arg-val-gly-tyr asx-leu-asx-gly-(ser,lys,glx,ser,ile,leu)

leu-gly-ala-arg-arg-val-gly-tyr-(gln,ile,leu)-asx-leu-asx-gly-ser-lys-(glx,ser,ile,leu)

Figure 25. Fragment 5.

P333 P244a

phe-arg-pro-glx-glx-arg-phe-ile-glu-arg-pro-(glu,gln)-leu-ser-arg-val-asp-ala-gly-glu-gln-leu-gly-gln-arg

T17a T6b

(C352c) has been isolated.

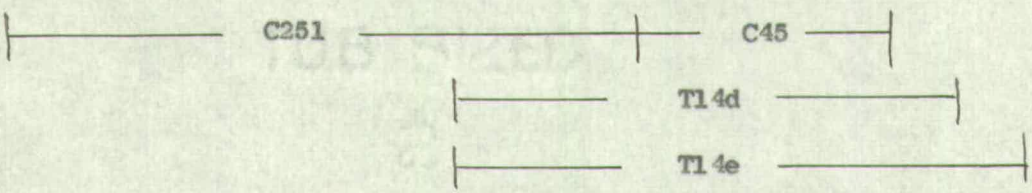
The peptides T18a and T8c were identified in tryptic sub-digests of the CNBr peptide X3 (Chapter VIII), which indicates that it is the N-terminal CNBr peptide. The observation that the fourth residue of X3 was arginine (Chapter VIII) is in accordance with the hypothesis that T18a and X3 are N-terminal peptides of β-lactamase.

The C-terminus of fragment 3 is tentatively overlapped by the peptic peptide P242 (Figure 24). The next tryptic peptide cannot be deduced.

Fragment 4 (16 residues)

The chymotryptic peptides C251 and C45 are overlapped by the tryptic peptides T14d and T14e.

glu-ser-phe-arg-pro-glu-arg-phe-pro-met-met-ser-thr-phe-lys-lys



T14d and T14e are correlated by the sequence of C45 and by the isolation of free homoserine (X712/X74) in the CNBr experiments. C251 [glu-ser-phe-arg-pro-glu-(arg,phe,pro,met)] was not completely sequenced but the composition of the unsequenced C-terminal region fits exactly in this fragment.

Fragment 5 (26 residues)

This fragment includes T17a and T6b together with P244a and P333 (Figure 25). The total sequence of T6b and the partial sequence of P244a agree well. The N-terminal region of P244a

correlates with the tentative C-terminal sequence of T17a and permits a total sequence to be deduced, assuming that P244a is produced by a peptic cleavage after a leucine residue. The sequence of P333 suggests that it overlaps with a tryptic peptide which has N-terminal phenylalanine. T14d and T14e are accounted for by fragment 4, and so an overlap between P333 and T17a is probable.

Fragment 6 (17 residues)

Fragment 6 is X6, the C-terminal CNBr peptide. It was discussed in Chapter VIII.

asp-glu-arg-asn-arg-glu-gln-ile-ala-ala-leu-gly-ser-ile-trp-lys-his

This peptide incorporates the peptic peptides P244b and P420; the interior of the sequence is less reliable because it is not correlated by an independently-isolated peptide. The sequence "-asn-arg-" probably gives rise to the tryptic peptide T18c (asn-arg) and T12a may represent the C-terminal tryptic peptide.

The partial and tentative sequence derived for peptide T16a suggests that it may overlap the N-terminus of X6 as shown in Figure 26. This tentative overlap makes up a partial sequence of 26 residues.

Peptide T17b

In the sequential degradation of T17b, the leucine and valine residues were not distinguished. The isolation of the tryptic sub-peptide thr-val-arg from X41 shows that the sequence of T17b is probably:

his-leu-asp-thr-gly-met-thr-val-arg

Figure 26. Tentative Overlap between T16a and X6

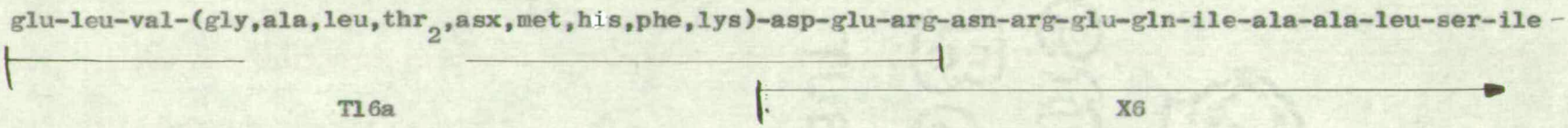
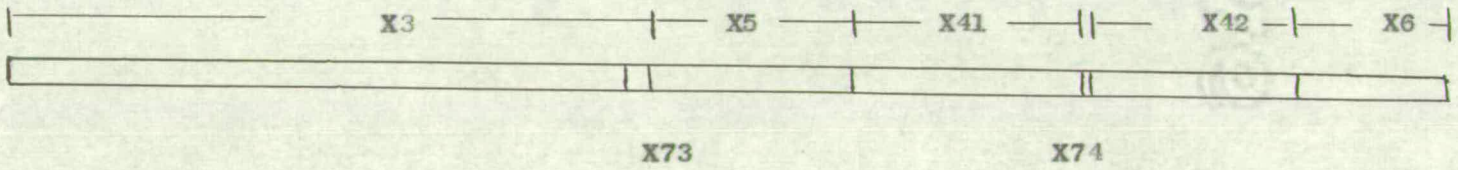


Figure 27. Tentative arrangement of CNBr Peptides



The Cysteine Residue

The determination of cysteine (Chapter II) and the "performic acid diagonal" experiment (Appendix III) suggests that there is a single cysteine residue in the β-lactamase molecule. It is difficult to reconcile what is known about C271, the cysteic acid-containing chymotryptic peptide with T2a, the corresponding tryptic peptide. The latter was impure and the former is at the present the only indication of the amino-acid sequence in the region of the cysteine residue of *E.coli* β-lactamase.

The Tryptophan Residues

Fragment 6 accounts for one tryptophan residue and peptide P412 (ala-pro-gly-try), which may be overlapped by peptide T11a, which also contains tryptophan, accounts for another. In the peptic digest, a potential third tryptophan-containing peptide was lost. The β-lactamase molecule contains three tryptophan residues (Chapter II).

Accounting For the Sequence

The well-characterised fragments account for about 110 residues and their tentative extensions bring this total to about 140 residues. Five well-characterised tryptic peptides are unaccounted for in these fragments:

T11b, T12b, T14a, T17b, T18c

These peptides total 44 residues and there are at least two poorly-

characterised tryptic peptides (T2a, containing the cysteine residue, and T2b) which must be included if the whole β-lactamase molecule is accounted for. The total of all of these is about 200 residues, but as there must be some overlaps between the fragments and other peptides, the real total is probably somewhat lower. Thus if the β-lactamase molecule contains about 180-190 amino-acids, it is quantitatively well accounted for by these fragments and the remaining peptides.

Further characterisation of some tryptic peptides, especially those from the C-terminal region and the cysteine-containing peptide, would be desirable for the completion of the sequence. More work with chymotryptic and peptic peptides might also be useful but in several instances the overlapping regions between these peptides and the tryptic peptides are short. Thus a proteolytic enzyme with a different specificity, such as thermolysin, might yield more useful information relating to the ordering of the tryptic peptides.

Cyanogen Bromide Peptides

An extremely tentative arrangement of the CNBr peptides within the E.coli β-lactamase peptide chain is shown in Figure 27. It is based upon the possibility that fragment 2, some tryptic peptides of which were isolated from sub-digests of X3, is adjacent to fragment 1, which overlaps X73 and X5. It is also based upon the fact that X6 is the C-terminal CNBr peptide and X3 is probably its N-terminal counterpart. A possible overlap between T17b and X42 was discussed earlier in the chapter, and this suggests that fragment 4 may overlap the C-terminus of X41, the free homoserine residue X712/X74 and the N-terminus of X42, as being the only CNBr peptide unaccounted for.

Homology Between β-Lactamase Sequences

Comparison of the tryptic peptides of E.coli β-lactamase with the amino-acid sequences of the S.aureus and B.licheniformis β-lactamases (Ambler and Meadway, 1969; Meadway, 1969b) yielded only two peptides which showed any sequence similarities. Peptide T17b is probably homologous with a tryptic peptide which corresponds to residues 84-92 in B.licheniformis β-lactamase.

his-leu-asp-thr-gly-met-thr-val-arg T17b

his-val-asp-thr-gly-met-thr-leu-lys

The corresponding peptide and sequence in S.aureus β-lactamase comprises residues 79-87:

tyr-val-gly-lys-asp-ile-thr-leu-lys

Peptide T2d was considered to be similar to the sequence comprising residues 151-162 in B.licheniformis β-lactamase and residues 146-157 in S.aureus β-lactamase.

Several large fragments, which comprised about 80% of the B.licheniformis β-lactamase sequence, were compared with the complete S.aureus β-lactamase sequence by means of a computer programme (Ambler and Meadway, 1969). In this way the homology between the sequences was demonstrated and confirmed. Although the fragments of the E.coli β-lactamase sequence reported here are less well-characterised and form a smaller proportion of the whole sequence, it was felt that a similar comparison between these fragments and the completed sequences would almost certainly reveal sequence homology if it exists.

Visual comparison of each of the six fragments described in

this Chapter with the completed sequences suggested that fragment 1, which contains T2d, might be partly homologous with the residues 134-162 in B.licheniformis β-lactamase and residues 129-157 in S.aureus β-lactamase. None of the other fragments could be successfully matched with similar regions of the completed sequences by visual comparison.

Fragments 1, 2, 5 and 6 were compared with S.aureus β-lactamase sequence using the same computer programme as was used by Ambler and Meadway (1969). The programme effectively slides the complete sequence past each fragment, residue by residue, noting the number of identical residues at each alignment and then printing out the psoition of alignment and the number of identical residues for each alignment at which a minimum number of identical residues is exceeded. In the present case, this minimum number was set at three.

All four fragments could be aligned in several positions relative to the complete sequence such that up to about 15% of the corresponding residues were identical. This must be considered as the level of "noise" due to chance matching of residues below which true sequence homology would not be detected in the present comparison.

Fragment 1 was shown to have two positions, with its N-terminal residue aligned with residues 129 and 130 respectively in S.aureus β-lactamase, at which this level of identity was slightly exceeded. If the deletion of a residue corresponding to lysine 144 or 145 is assumed, then fragment 1 can be aligned with S.aureus β-lactamase so that 10 residues in the 29-residue fragment are identical and so that there are several "conservative substitutions". A similar

alignment relative to B.licheniformis β-lactamase is also possible. These alignments are shown in Figure 28.

This phenomenon must be viewed with caution. The homology between fragment 1 and the completed sequences may be an artefact of the "-ala-ala-" and "-thr-thr-" sequences. The N-terminal region of fragment 1 has not been completely sequenced. Fragment 2 is not homologous with a region of the completed sequences such that it is N-terminal to fragment 1.

The amino-acid sequences of the S.aureus and B-licheniformis β-lactamases have identical amino-acid residues in 105 of the 257 possible positions. These residues tend to be in short sequences of a few residues, distributed throughout the molecules. The available evidence suggests that if the E.coli β-lactamase sequence is similarly homologous, then this homology is no more than 15% i.e. there will be no more than 25-30 identical residues in a sequence of 180 when the full sequence is compared with those already determined. The correct positioning of a fragment of a complete sequence relative to another complete sequence on the basis of homology is reinforced by the existence of the rest of the sequence and so the "noise" level of such a comparison will be effectively lower. Nevertheless, such a slight degree of homology will be difficult to demonstrate conclusively.

The peptide T17b, and possibly fragment 1, are regions of the E.coli β-lactamase sequence which are very similar to regions of the complete sequences and other similar regions may yet be discovered. Comparison of T17b with the corresponding B.licheniformis sequence suggests that this may be a strongly-conserved region of sequence but this is not borne out when the corresponding S.aureus sequence is considered.

Figure 28.

pro-val-arg-tyr-glu-ile-glu-leu-asn-tyr-tyr-ser-pro-lys-ser-lys-lys-asp-thr-ser-thr-pro-ala-ala-phe-gly-lys-thr-leu-asn

129 157

S.aureus

leu-asp-arg-glx-glx-pro-asx-leu-glu-pro-asn-ala-(asx,glx,ile)-arg asp-thr-thr-met-pro-ala-ala-met-ala-thr-thr-leu-arg

fragment 1

pro-glu-arg-phe-glu-pro-glu-leu-asn-asp-val-asn-pro-gly-glu-thr-gln-asp-thr-ser-thr-ala-arg-ala-leu-val-thr-ser-leu-arg

135 163

B.licheniformis

CHAPTER X.

RELATIONSHIPS BETWEEN STRUCTURE AND FUNCTION IN *E.COLI* β-LACTAMASE

The sequence studies on *E.coli* β-lactamase have shown that there is likely to be very little homology between the full sequence of this protein and the sequences of the *B.licheniformis* and *S.aureus* β-lactamases. The few structural similarities that do exist may represent a residual degree of sequence homology between proteins with a distant common ancestor, i.e. divergent evolution, or they may represent the essential elements in the structure of a β-lactamase acquired by mutation and natural selection, i.e. convergent evolution. Chemical modification and enzymological studies (reviewed in Chapter I) on the Gram-positive β-lactamases have given some insight into the relationship between structure and function in these enzymes. This chapter describes experiments which were carried out in order to obtain a similar insight into *E.coli* β-lactamase. It was hoped that a comparison of the β-lactamases in this respect might clarify their evolutionary relationships.

Methods

The spectrophotometric variant of the Perret (1954) assay for β-lactamase developed by Sherratt and Collins was used for the chemical modification studies (see Chapter II for references and description). Michaelis parameters were determined using the micro-iodometric assay (Novick, 1962; see Chapter II). The variation of these parameters with pH was studied using 0.1 M sodium phosphate buffers (Gomori, 1955).

β-lactamase (0.1 μmol in 10 ml 0.005 M-Tris-HCl pH 7.5) was incubated with diisopropylfluorophosphate (0.005 ml DFP in 0.5 ml isopropanol) for 1 h at room temperature in a screw-cap flask (Cohen et al., 1969). It was assayed for β-lactamase activity at 0, 30 and 60 min. There was no detectable loss of activity. DFP is extremely toxic and large volumes of 1 M-NaOH in 50% ethanol were used to detoxify used glassware and residual solutions.

Tetranitromethane was first demonstrated as a tyrosine-specific modification reagent by Sokolovsky et al., (1966). The reaction conditions, possible side-effects and examples of the nitration of tyrosine residues involved in the activity of various enzymes have been described (Riordan and Vallee, 1972).

0.10 ml of TNM* was added to 4.2 ml of 95% ethanol to form a 20 mM solution, which was further diluted as required. TNM is both toxic and explosive. Large volumes of 1 M-ammonia were kept at hand for the detoxification of glassware and residual solutions.

Trial experiments indicated that incubation of E.coli β-lactamase with TNM at pH 8.0 caused a rapid, partial loss of activity. Molar excesses of TNM from 10-fold to 100-fold, and incubation times from 1-3 h all caused a loss of approximately 40% of the enzymic activity. Other experiments indicated that the rate of loss of activity was independent of the concentration of TNM, within the same range. E.coli β-lactamase activity was not affected by incubation with TNM at pH 6.8.

To one μmol of β-lactamase in 40 ml of 0.1 M-sodium phosphate buffer pH 8.0 was added 10 μmol of TNM in 0.05 ml 95% ethanol. After 1 h at room temperature the loss of activity was 36%. The solution was freeze-dried, desalted on a small column of Sephadex

* Ralph N. Emanuel.

G-25 in 0.1 M-ammonia and freeze-dried again. It was then incubated with 0.5 mg each of DPCC-trypsin and SBTI-chymotrypsin for 4 h at 37°C. A single yellow band was purified from the digest by paper electrophoresis at pH 6.5 and pH 3.5.

Two smaller batches of nitro-β-lactamase were also prepared, one of which was digested with DPCC-trypsin.

Photooxidation of specific residues in proteins was successfully exploited in a quantitative fashion by Weil and co-workers. Irradiation with visible light of protein solutions containing traces of methylene blue caused the loss of biological activity which could be correlated with the destruction of specific amino-acid residues. (Weil et al., 1951; 1953).

A simple experimental technique was used for the photooxidation of β-lactamase (Figure 29). The protein solution was added to a small beaker with a magnetic stirring bar. It was mounted in a shallow glass dish containing ice-water on a magnetic stirrer. Irradiation was with a desk lamp fitted with a 15 W tungsten-filament bulb. The shade of the lamp served to exclude external light and to maintain the light source at a fixed distance from the protein solution. Methylene blue solution was added, after a pre-incubation under the light source to stabilise the temperature. The temperature was fairly stable at 35-40°C in control experiments. Samples were taken with a pipette. β-lactamase assays were performed immediately and samples were stored in the dark prior to desiccation and hydrolysis for amino-acid analysis. A similar method has been used by Kenkare and Richards (1966).

In one experiment, 0.5 μmol of β-lactamase in 10 ml 0.05 M-sodium phosphate pH 7.0 containing 0.01% methylene blue was

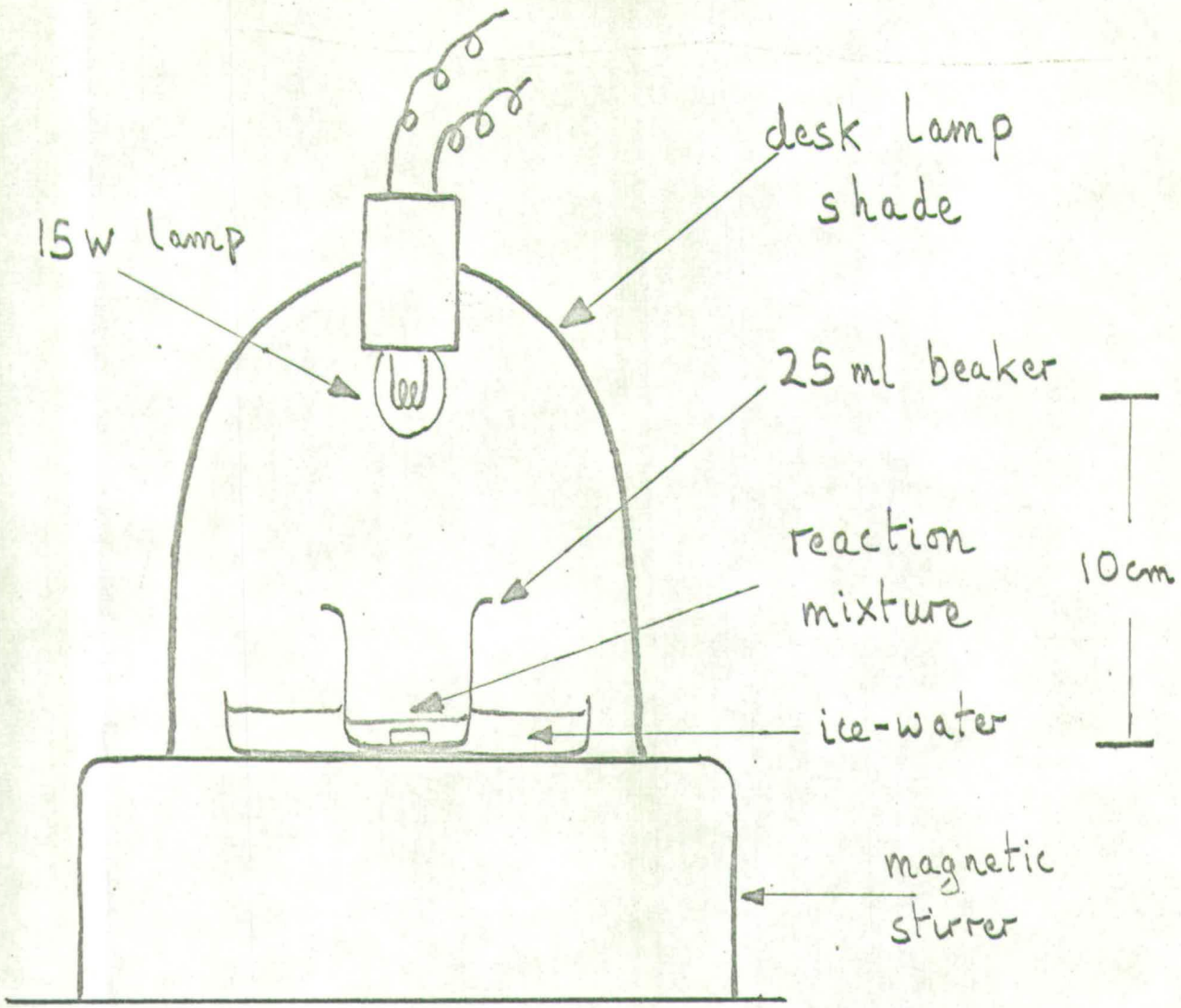


FIGURE 29 PHOTOOXIDATION APPARATUS

irradiated for 1 h with a 15 W source at a distance of 10 cm. At 15 min intervals samples were taken for amino-acid analysis and these were also assayed for β-lactamase activity. At the end of the experiment, less than 5% of this activity remained.

At pH values near to neutrality the carboxymethylation of histidine residues is often the most rapid reaction of iodoacetic acid with proteins (Gundlach et al., 1959; Gurd, 1967).

β-lactamase solutions of about 2×10^4 units/ml in 0.1 M-sodium phosphate buffer pH 7.0 were mixed with equal volumes of the same buffer containing various concentrations of IAA (0.05-1 mg/ml) and incubated at room temperature. The β-lactamase activities of the mixutres were assayed at intervals for 8 h, and a final assay was made after 20 h.

Following this experiment, 0.5 μmol of β-lactamase in 5 ml 0.1 M-sodium phosphate pH 7.0 was mixed with 5 mmol of ^{14}C-IAA* (0.015 mc per mmol) dissolved in 1 ml of the same buffer. After incubation for 2 h at room temperature, the preparation was desalted on a small column of Sephadex G-25 in 0.1 M-ammonia, freeze-dried and then digested for 4 h at 37°C with 0.25 mg of DPCC-trypsin.

The digest was fractionated by paper electrophoresis at pH 6.5 and pH 3.5 and the peptides containing modified residues were identified by autoradiography. Electrophoretograms were incubated in contact with photographic films (Kodak Blue Band) in the dark for two or three weeks. One such autoradiograph is shown in Figure 33.

Results

A single serine residue has been implicated in the enzymic

---

* The Radiochemical Centre.

activity of many proteases and esterases (Milstein and Sanger, 1961) and the inactivation of these enzymes by the phosphorylation of the serine residue with organophosphorus compounds such as DFP is a notable common characteristic (Koshland, 1959). DFP does not inactivate E.coli β-lactamase, which suggests, but does not prove, that serine residues are not involved in the active site of this enzyme.

Experiments with Tetranitromethane

The results of preliminary experiments, considered in the light of the specific nitration of a single tyrosine residue in B. licheniformis β-lactamase (Meadway, 1969a,b) suggested that a similarly specific reaction might occur with E.coli β-lactamase.

Samples of the enzyme [redacted] hydrolysed and analysed for amino-acids before and after reaction with TNM showed that the 36% loss in activity was accompanied by the loss of 32% of the original tyrosine content and by the recovery of a corresponding amount of 3-nitrotyrosine.

It has been shown that TNM will oxidise sulphydryl groups in proteins (Sokolovsky et al., 1969). At low pH values, TNM will not react with tyrosine and is specific for cysteine residues. No loss of cysteine was observed as a result of the reaction of TNM with β-lactamase at pH 8.0. At pH 6.8, TNM did not inactivate the enzyme. If cysteine was oxidised slowly, it would have been difficult to detect in these experiments, but if so, there was no detectable loss of enzymic activity associated with it.

A single peptide containing 3-nitrotyrosine was isolated from a tryptic and chymotryptic digest of nitro-β-lactamase. The

composition was:

asx	2.0	(2)
thr	0.2	
ser	1.0	(1)
glx	2.1	(2)
gly	0.2	
ala	0.2	
val	0.9	(1)
leu	0.9	(1)
$tyrNO_2$	0.9	(1)

The N-terminus was serine and the mobility at pH 6.5 was -0.6. In these respects, the peptide is identical with the chymotryptic peptide C392a [ser-glx-asx-val-(asx,glx,leu,tyr)] except that it contains 3-nitrotyrosine instead of tyrosine.

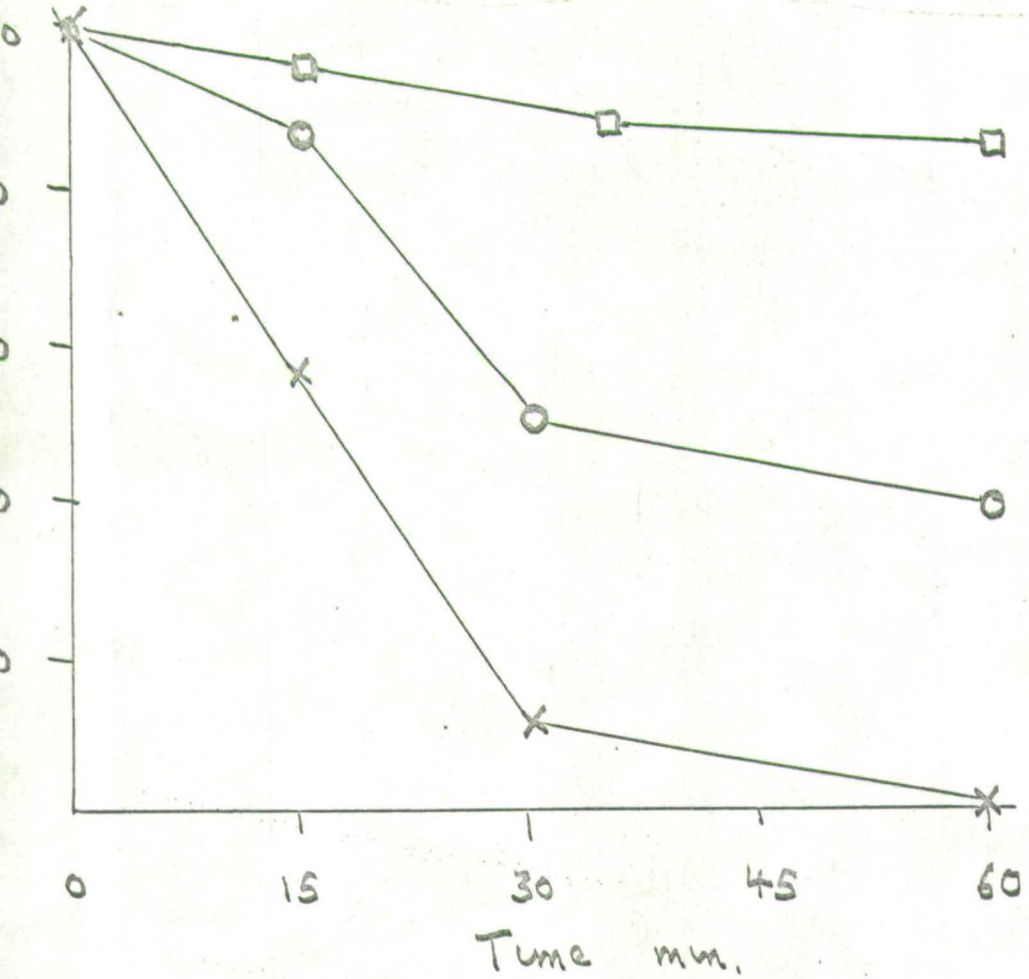
A second sample of nitro-β-lactamase was digested with trypsin and a single yellow band was purified by paper chromatography and electrophoresis. A large, impure, neutral peptide with N-terminal leucine was isolated but a poor amino-acid analyser run rendered an uninterpretable analysis.

Tetranitromethane reacts with E.coli β-lactamase to cause a partial loss of catalytic activity due to the nitration of a single tyrosine residue which is contained in the chymotryptic peptide C392a.

Photooxidation of E.coli β-lactamase

Figure 30 shows the decay of β-lactamase activity during photooxidation. Samples taken at intervals were hydrolysed in vacuo with 6 M-HCl for 24 h and analysed for amino-acids. The

igure 30 Photooxidation of β-Lactamase



× Residual activity as % of initial activity

○ Residual histidine content, as " " " content

□ Residual methionine " , " " " " "

destruction of half of the total histidine content accompanied the loss of activity. About 12% of the methionine content was also destroyed but it has been shown that the photooxidation of methionine produces methionine sulphoxide which reverts almost quantitatively to methionine under the conditions of acid hydrolysis (Weil et al., 1951). There was no photooxidative destruction of any other amino-acids, although the tryptophan contents were not measured.

A second experiment confirmed that the destruction of half of the histidine content of β-lactamase (i.e. 2 out of 4 residues) was approximately paralleled by the loss of enzymic activity and that tyrosine in the protein was not affected by photooxidation. Simultaneously, samples were taken for alkaline hydrolysis in order to study the methionine and tryptophan contents of the enzyme during photooxidation (Noltman et al., 1962). As was noted in Chapter IV, the recoveries of the various amino-acids were variable.

The entire photooxidised β-lactamase from a third experiment (0.5 μmol) was desalted on a small column of Sephadex G-25 in 0.1 M-ammonia and digested with 0.25 mg DPCC-trypsin, in parallel with 0.5 μmol of the native enzyme. Part of each digest was subjected to pH 6.5 electrophoresis in parallel. The electrophoretogram was stained with the Pauli reagent, which is specific for tyrosine and histidine. The "photooxidised" strip gave very faint spots. The only spot not present in the "photooxidised" strip, relative to the "native" strip was at mobility +0.1.

The experiments involving the photooxidation of E.coli β-lactamase showed that one or more histidine residues were probably involved in the catalytic activity of the enzyme. Consideration of the compositions and mobilities of the tryptic peptides (see

Table 10 in Chapter V) suggested that T17b and/or T17c may be peptides which contain a histidine residue essential to the activity of β-lactamase. It is possible that the loss of activity on photooxidation was partly or wholly due to the oxidation of one or more methionine residues to methionine sulphoxide, and that reversion of this compound to methionine during acid hydrolysis masked this effect.

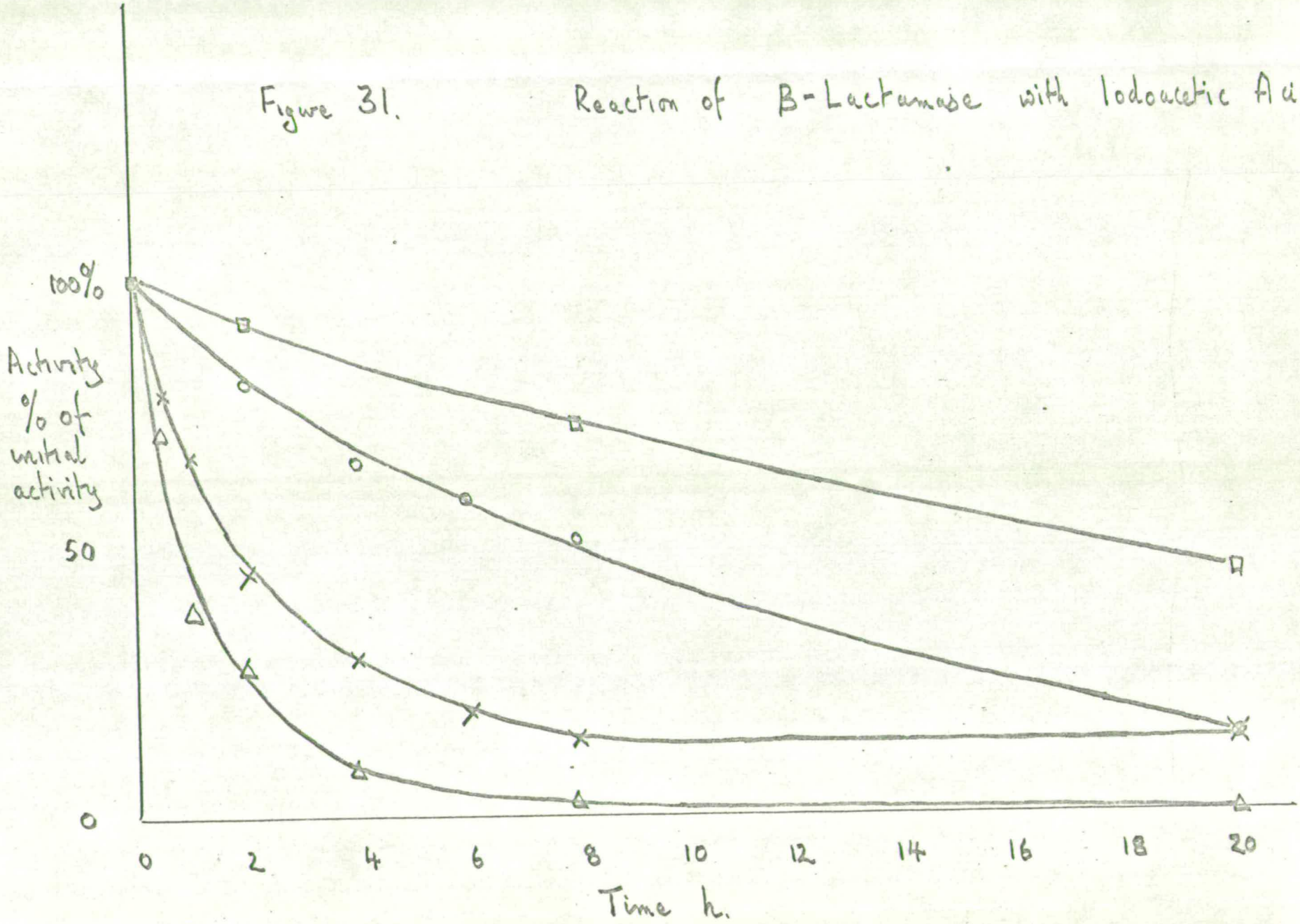
Ray and Koshland (1960, 1962) have undertaken kinetic studies of photooxidation. The sum of the first order rate constants for the photooxidative destruction of certain residues was found to equal the first order rate constant for the loss of biological activity. This technique precludes the possibility of the wrong assignment of residues to the active site because of their fortuitous photooxidation. It was considered that the application of this technique required data of greater precision than was possible with the simple experimental technique used in the present work.

At this point, experiments with iodoacetic acid were initiated, as it was considered that isolating and analysing peptides containing modified residues would be easier than proving that certain peptides were not present after photooxidation.

Experiments with Iodoacetic Acid

Figure 31 shows the decrease in enzymic activity with time when β-lactamase was incubated at pH 7.0 with various concentrations of IAA ([IAA]). At each concentration of IAA, the initial rates of inactivation of β-lactamase (V_o) were measured and Figure 32 shows ($- \log_{10} V_o$) plotted against ($\log_{10}$[IAA]). A straight line

Figure 31. Reaction of β-Lactamase with Iodoacetic Acid.



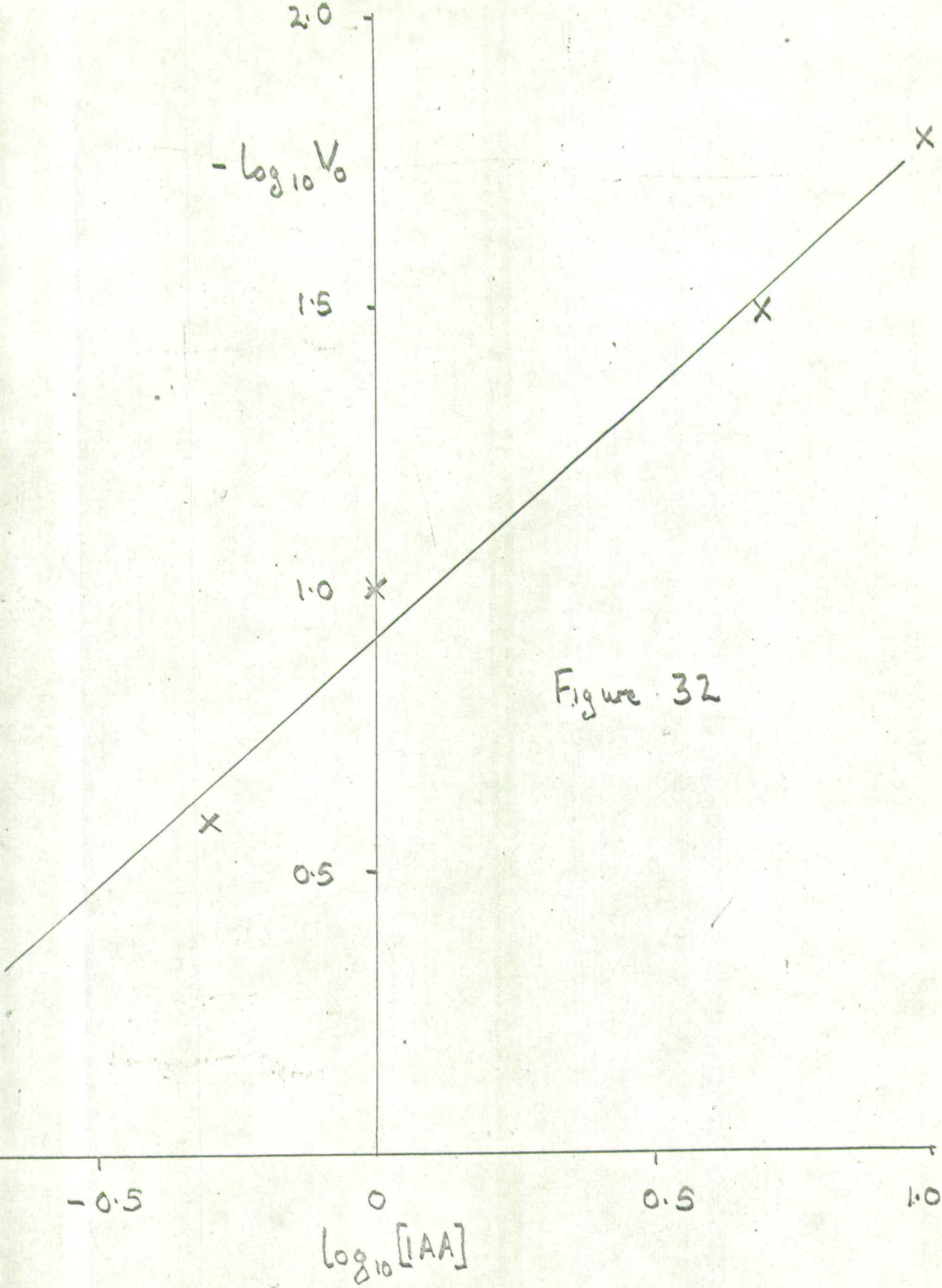


Figure 32

of slope +0.95 was obtained, indicating that the inactivation of β-lactamase by IAA is first order in IAA. Order does not necessarily equate with molecularity but the simplest explanation of these results is that a single molecule of IAA reacts with a single molecule of β-lactamase to inactivate it. IAA may react with β-lactamase in other ways which do not affect the activity of the enzyme.

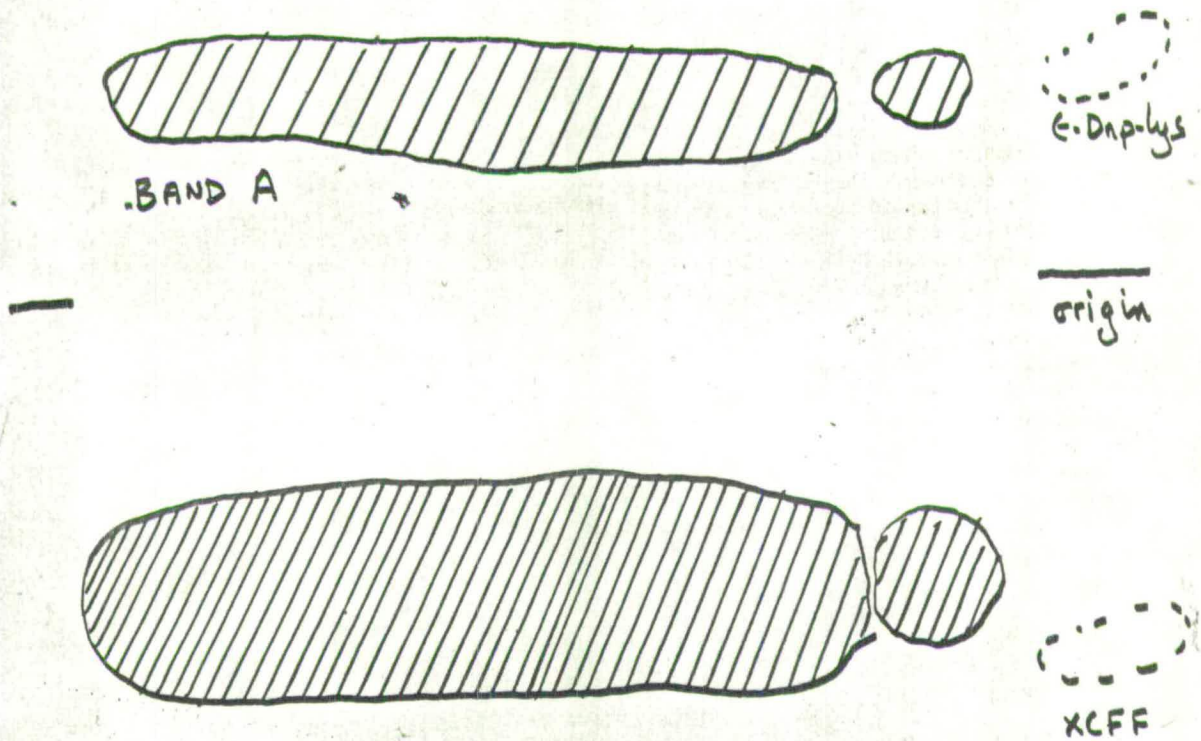
Reaction of β-lactamase with a 10^4-fold molar excess of IAA at pH 7.0 for 4 h caused an 85% loss of activity and a 35% loss of histidine.

0.5 μmol of β-lactamase was reacted with 5 mmol of IAA at pH 7.0 for 2 h. It was digested with trypsin and Figure 33 shows the autoradiograph prepared from the preparative pH 6.5 electrophoretogram of the digest.

The three radioactive bands were eluted and further purified by pH 3.5 electrophoresis. Only the band B contained detectable amounts of ninhydrin-positive material. A single, pure peptide was isolated in low yield. Its composition corresponded to:

asx	:	1.0	(1)
thr	:	1.8	(2)
ser	:	0.5	
CM-his	:	1.1	(1)
gly	:	1.0	(1)
val	:	1.0	(1)
met	:	0.9	(1)
leu	:	1.0	(1)
arg	:	1.1	(1)

FIGURE 33 TRACING OF AUTORADIOGRAPH



The peak tentatively identified as carboxymethyl-histidine eluted in a position between the expected positions of glutamic acid and proline, and had a small shoulder on the trailing edge. The quantity of CM-hisidine was calculated using the colour constant for glutamic acid. The experiments of Crestfield et al. (1963) on the alkylation of histidine residues in ribonuclease suggests that this was 1-carboxymethyl-histidine.

These experiments show that the inactivation of E.coli β-lactamase by IAA at pH 7.0 is due to the specific alkylation of a single histidine residue which corresponds to the N-terminal residue of peptide T17b.

A similar specificity of alkylation of histidine residues in ribonuclease was observed by Moore and Stein and co-workers (Crestfield et al., 1963; Heinrikson et al., 1965), who also noted that the alkylation was rapid relative to the alkylation of N-acetyl histidine under the same conditions. Presumably the environment of the susceptible histidine residue in β-lactamase is such as to promote rapid alkylation as the C-terminal histidine residue would be expected to be exposed to the solvent. Prolonged treatment with IAA resulted in the alkylation of more than one histidine residue and the 2 h incubation used in the preparative experiment was chosen so as to permit the isolation of a single labelled peptide. The two minor bands in the autoradiograph may have been the result of the alkylation of other residues, although they may also have been due to the formation of a 1,3-dicarboxymethylhistidine peptide, or to the partial proteolytic cleavage of peptide bonds.

No CM-cysteine was observed in the analyses of β-lactamase treated with IAA under the conditions described here. At pH 8.3

in 6 M urea, a 2 h incubation with a large excess of IAA resulted in the formation of 0.9 mols of CM-cysteine per mol of β-lactamase. Under the same conditions about 40% (1.4 mol/mol) of the histidine content of β-lactamase disappeared (Chapter II).

The experiments with TNM and IAA suggest that the single cysteine residue in E.coli β-lactamase is unreactive and may be shielded in the interior of the molecule. However, Datta and Richmond (1966) observed a loss of 15% of the β-lactamase activity when the enzyme was incubated with 1 mM-p-chloromercuribenzoate for 15 min at room temperature. The β-lactamase isolated from Aerobacter cloacae 53 was shown to contain 2 or 3 mols cysteine/mol and to be considerably more sensitive to p-chloromercuribenzoate (Jack, 1971).

The main limitation of these studies is that it is impossible to be sure that the modified residue is at the active site of the enzyme. An attempt to use a substrate or inhibitor of β-lactamase to block chemical modification is impractical since even the best inhibitors are hydrolysed very rapidly (Meadway, 1969a).

Variation of β-lactamase Activity with pH

Michaelis parameters K_m and V_{max} for β-lactamase were calculated over a range of pH values from values of the initial rate of hydrolysis of various concentrations of sodium benzylpenicillin (Lineweaver and Burk, 1934). The results, together with values of pK_m, Log V and $-[\log V/pK_m]$, are shown in Table 22. K_m is defined as the substrate concentration at which the enzyme attains one half of its maximal initial rate V, which is also the molecular activity and is expressed in mols of substrate hydrolysed per mol enzyme per minute.

Table 22. Variation of Michaelis Parameters with pH

pH	K_m	pK_m	V	log V	$-[\log V/pK_m]$
5.85	29 μM	5.64	1.58×10^4	4.20	1.46
6.46	17 μM	5.77	1.67×10^4	4.22	1.55
6.90	12 μM	5.92	1.85×10^4	4.27	1.65
7.10	31 μM	5.51	1.51×10^4	4.18	1.33
7.49	40 μM	5.40	0.94×10^4	3.97	1.43
7.72	100 μM	5.00	0.58×10^4	3.76	1.34
8.00	220 μM	4.64	0.27×10^4	3.43	1.21

The logarithmic functions are plotted against the pH in Figure 34. Also shown in Figure 34 is a plot of log V against pH for nitro-β-lactamase. In this case, the V at each pH was measured using the spectrophotometric Perret assay and expressed as a percentage of V at pH 6.90.

Dixon (1953; reiterated and expanded by Dixon and Webb, 1964) has developed a theory which interprets the effect of pH on the kinetics of enzymic reactions in terms of the ionisation of groups in the enzyme, the substrate and the enzyme-substrate complex. From the Michaelis-Mentin equation and the Michaelis pH functions is derived an equation:

$$pK_s = pK_s^{'} - pf_{es}^{'} + pf_e^{'} + pf_s$$

K_s is the substrate constant derived from Michaelis-Mentin equation and $K_s^{'}$ is the equilibrium constant for the reaction between the reactive ionic species of the enzyme and the substrate. The Michaelis pH functions ($f_{es}^{'}$, $f_e^{'}$ and f_s) define the amount of each ionic species of the enzyme, the substrate and the enzyme-substrate

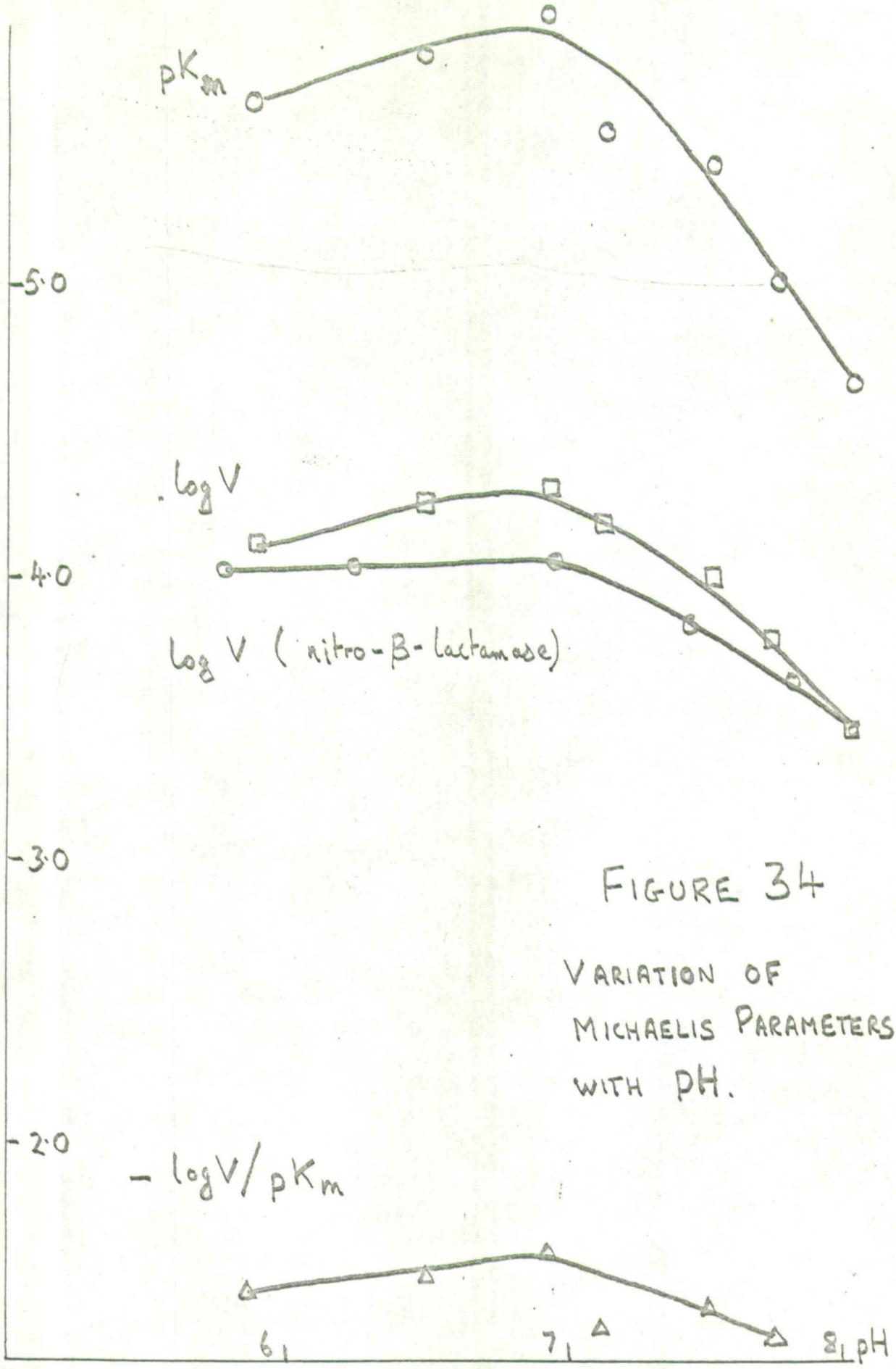


FIGURE 34

VARIATION OF MICHAELIS PARAMETERS WITH pH.

complex in terms of the total amount of each, respectively. If it is assumed that the theoretically-derived substrate constant K_s can be equated with the operationally-defined Michaelis constant K_m, then this equation can be used to interpret graphs of pK_m plotted against pH.

pK_s' is a constant term and merely affects the vertical position of the graph, whilst the other terms are all functions of the degree of ionisation of the enzyme, the substrate and the enzyme-substrate complex. The graph will consist of straight regions joined by short, curved sections each of which denote an ionisation in one of the components. Extrapolation of the straight regions on either side will intersect at the pH corresponding to the pK of the ionising group. Each ionisation of a group in the enzyme-substrate complex will increase the gradient by +1 and each ionisation in the enzyme or the substrate will decrease the gradient by -1. The gradient in any portion will be equal to the change in charge occurring when the enzyme-substrate complex dissociates into free enzyme and substrate.

A similar treatment of plots of log V *vs* pH is possible. The pKs of groups in the enzyme-substrate complex can be identified in this way.

Dixon (1953) has pointed out that the pKs of the ionisations of amino-acid side chains in proteins often differ considerably from those in the free amino-acids. In addition, the pKs determined are molecular ionisation constants rather than group ionisation constants. The assumption that $K_s = K_m$ may not be valid. Cleland (1970) has emphasised that the overall interaction between enzyme and substrate may be complex and that the rate-determining step may

change with pH. For these reasons the identification of amino-acids involved in the activity of an enzyme on the basis of pKs alone is unreliable.

In order to partially avoid this limitation, Cleland (1970) has advocated the plotting of $-[\log V/pK_m]$ *vs* pH as being effectively a plot of log [binding] *vs* pH. The binding of the enzyme and the substrate is a one-step process and any pKs will be true pKs.

A consideration of the results of the chemical modification studies of *E.coli* β-lactamase permits an interpretation of the data in Figure 34 despite these limitations.

Sodium benzylpenicillin does not ionise in the pH range studied. There appear to be two groups which ionise in both the enzyme and the enzyme-substrate complex and which have pKs close to 7. Ionisation of one of these groups causes a total loss of activity, represented by the gradient of -1 on the right-hand-side of the graph.

The left-hand-side of the graph has a slope of approximately + 1/3 and, following the interpretation of Cleland (1970), this represents the protonation of a group, causing a partial loss of activity. This phenomenon is eliminated in nitro-β-lactamase. This suggests that the tyrosine residue which is susceptible to nitration is required to be unprotonated for full activity. This may explain the partial loss of activity which accompanies nitration. The fact that TNM is so specific argues for an abnormally low pK for this residue (Riordan and Vallee, 1972). If this is so, then the reactive histidine residue may be the residue which is required to be protonated for full activity. Ionisation, or chemical modification, inhibits the activity of the enzyme

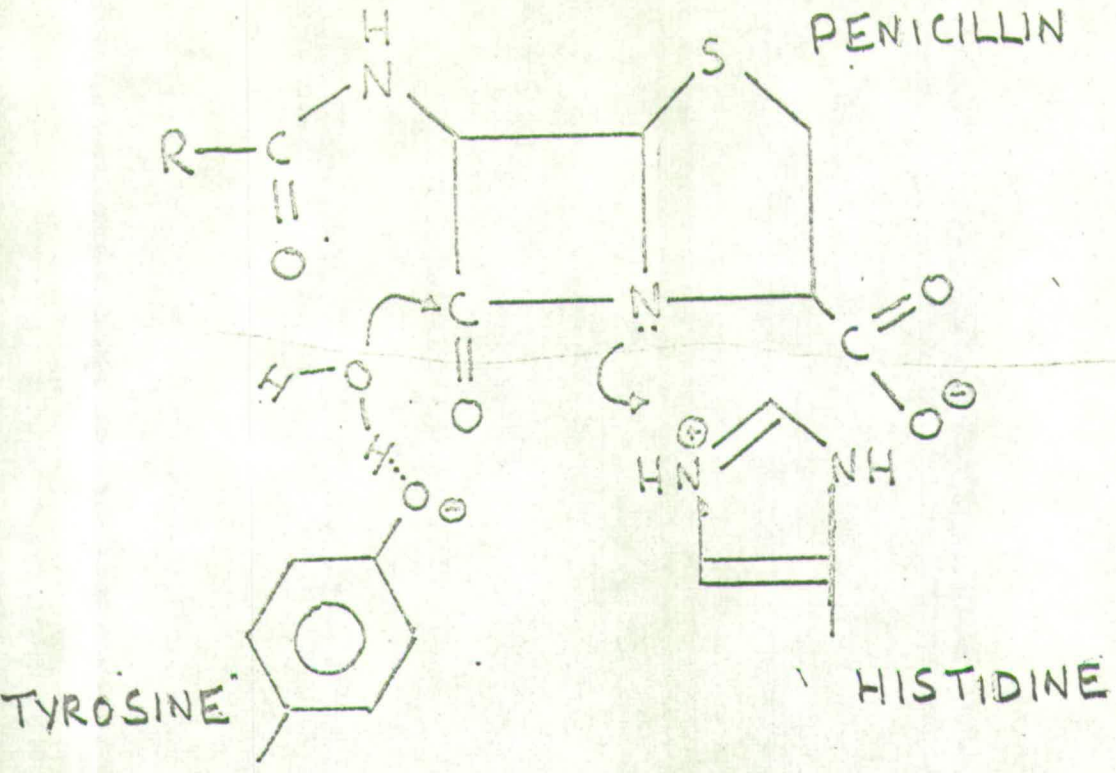
completely. These ionising groups have little or no effect upon the binding between the enzyme and the substrate.

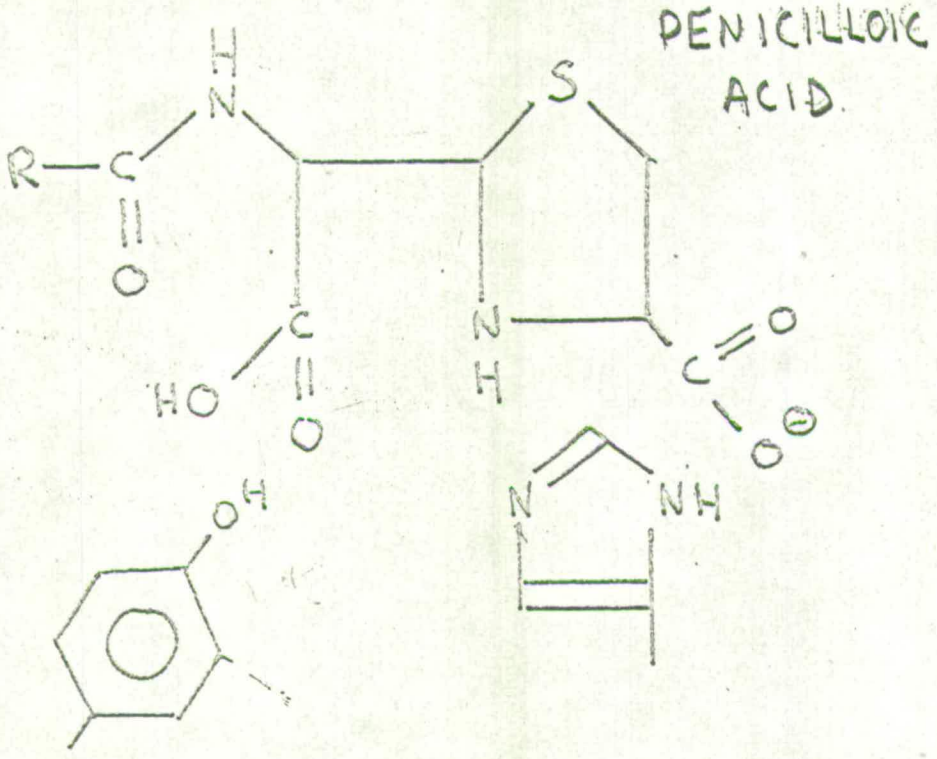
Proposed Mechanism of *E.coli* β-lactamase

The molecular activity of *E.coli* β-lactamase is 6×10^4 mols/mol/min, i.e. 10^3 mol/mol/sec, which is near the upper limit for a reaction in which proton transfer is rate-limiting (Eigen and Hammes, 1963), and suggests that a covalent reaction intermediate is unlikely. The activation energies for the enzymic hydrolysis of penicillin range from 3-8 kcal/mol (Smith and Hamilton-Miller, 1963). The hydrolysis of p-nitrophenyl acetate is catalysed by imidazole and acetylimidazole is an intermediate (Bruice and Schmir, 1957). The activation energy of this reaction is about 16 kcal/mol. Thus a penicilloate ester of the imidazole moiety of histidine is an unlikely reaction intermediate.

On the basis of the experimental work described earlier in this chapter and the interpretation thereof, a tentative mechanism for the catalysis of penicillin hydrolysis by β-lactamase is proposed (Figure 35). The histidine residue serves to protonate the β-lactam nitrogen in penicillin. The phenoxide ion of the tyrosine residue polarises a water molecule, promoting nucleophilic attack by hydroxyl ion at the β-lactam carbon atom and possible then serving to reprotonate the imidazole moiety.

This mechanism is both tentative and partial. It explains the experimental observations in the simplest way. It is possible that the histidine and tyrosine residues are not at the active site, that the observed ionisations are of other groups and hence that this interpretation is founded on false assumptions. It is also


PENICILLIN
TYROSINE
HISTIDINE


PENICILLOIC
ACID

possible that this interpretation is too simple. An examination of the reactivity of the cysteine residue and of tryptophan residues in β-lactamase might introduce new data which might modify or nullify the proposed mechanism. Probably there is a positively-charged residue at or near the β-lactamase active site which serves to bind the substrate through its carboxyl group to the enzyme. The observation that the β-lactam of 1,1-dimethyl-(2-methylthiazolidin-2-yl) acetic acid, which has the structure of the penicillin nucleus without the carboxyl group, is not hydrolysed by β-lactamase may imply an ionic interaction of this nature (Pollock, 1957).

The different relative activities with different penicillins and cephalosporins (see Chapter I) are presumably due to the effects of interactions between the different side-chains and the β-lactamase molecule.

Thus there is a need for further chemical modification and enzymological study of β-lactamase. The final test of the validity of this mechanism could be the determination of the tertiary structure of the enzyme by X-ray crystallography.

If this mechanism is used as a working hypothesis, then it predicts that the reactive tyrosine and histidine residues are likely to be conserved in an evolving protein sequence. The reactive tyrosine-containing peptide in E.coli β-lactamase is C392a :

ser-glx-asx-val-(glx,asx,leu,tyr)

The corresponding tyrosine-containing peptides in the B.licheniformis, S.aureus and B.cereus β-lactamases are:

B.licheniformis	asp-asp-leu-val-asn-tyr
S.aureus	asp-asp-ile-val-ala-tyr
B.cereus	glu-asp-leu-val-asn-tyr

(Meadway, 1969b; Ambler and Meadway, 1969; Thatcher, personal communication)

The E.coli peptide is a chymotryptic peptide whereas the others are produced by tryptic and chymotryptic cleavage. The three sequences from Gram-positive organisms are quite similar to each other whereas the E.coli sequence is somewhat different.

In contrast, the histidine-containing peptide in E.coli β-lactamase is very similar to sequences in the B.licheniformis and B.cereus β-lactamases, whereas the S.aureus β-lactamase sequence does not have a histidine residue in the corresponding position.

B.licheniformis	his-val-asp-thr-gly-met-thr-leu-lys
B.cereus	his-val-asp-thr-gly-met-lys
E.coli	his-leu-asp-thr-gly-met-thr-val-arg
S.aureus	tyr-val-gly-lys-asp-ile-thr-leu-lys

It is possible to envisage a tyrosine residue replacing the histidine residue in the proposed mechanism. If this were the case in S.aureus β-lactamase then there would be two tyrosine residues with abnormally low pKs and it is possible that they would both be susceptible to nitration by TNM. This was not observed in the experiments with TNM and S.aureus β-lactamase (R.P. Ambler, personal communication). Another histidine residue in this molecule may be reactive. Further experiments with this enzyme may give new insight into this problem.

The activity *vs* pH curves of the *B.licheniformis*, *S.aureus* and *E.coli* β-lactamases are similar, with a fairly broad pH optimum around neutrality, with a very steep fall on the alkaline side and a gentler fall on the acid side (Pollock, 1965; Richmond, 1963; Datta and Richmond, 1966).

There are chemical and enzymological similarities amongst the various β-lactamases which must be considered in the evaluation of the evolutionary relationships between these enzymes.

CHAPTER XI.

DISCUSSION

An improved procedure resulting in the 430-fold purification of the β-lactamase from Escherichia coli W3310 containing the TEM resistance transfer factor (Datta and Kontomichalou, 1965) has been described. Recoveries of over 60% and specific activities of 18.5×10^4 units of β-lactamase activity per milligram of protein (3.1×10^3 International Units per milligram) have been routinely achieved. This specific activity is greater than the value of 8.0×10^4 units per mg reported by Datta and Richmond (1966). The purification procedure is straightforward and reproducible and yields of 15 milligrams of pure protein per litre of bacterial culture are possible (Appendix I). The relative activities with respect to various penicillins and cephalosporins (the substrate profile) and electrophoretic mobility of the purified enzyme are identical with the β-lactamase purified from E.coli TEM by Datta and Richmond (1966). The identity of β-lactamases specified by the same RTFs but purified from different strains and species of bacteria has been demonstrated (Neu and Winschell, 1970; Dale and Smith, 1971a).

An estimation of the degree of purity of a protein preparation must be based upon several distinct criteria and even then it is limited by the sensitivity of the analytical methods employed.

The β-lactamase prepared by the present method was considered to be homogeneous by the criteria of starch gel electrophoresis at pH 8.5 and pH 4.0, by rechromatography and by gel filtration at pH 7.0 and in 50% formic acid. It was estimated that the starch

gel electrophoresis technique would detect a contaminating protein amounting to 4% or more of the β-lactamase protein.

A very faint, low-molecular-weight contaminant was observed when β-lactamase was subjected to SDS-polyacrylamide gel electrophoresis. From the experiments of Weber and Osborn (1969), it is estimated that 0.001 mg of protein could be detected by this technique. This represents about 5% of the sample of β-lactamase applied to the gels. Thus the β-lactamase was not absolutely homogeneous.

Various experiments with different preparations of β-lactamase confirmed the purity of the protein. The variation of the results of amino-acid analyses on samples of β-lactamase from different preparations were within the limits of accuracy of amino-acid analysis. The investigations of the N-terminus using 2,4-fluorodinitrobenzene and of the C-terminus using carboxypeptidase A also suggested that the protein was pure.

It was considered that E.coli β-lactamase prepared by this technique was sufficiently pure to be used in amino-acid sequence studies.

The two determinations of the molecular weight of E.coli β-lactamase by physical methods gave values of 2.1×10^4 and 2.7×10^4 daltons. An earlier determination gave a value of 1.7×10^4 daltons (Datta and Richmond, 1965). The limitations of each of these determinations were discussed in Chapter II. The only deduction that can be made from these results is that the amino-acid analyses of E.coli β-lactamase should be interpreted in terms of a molecular weight of $2.0 \times 10^4 - 2.5 \times 10^4$ daltons, rather than of 1.5×10^4 or 3.0×10^4 daltons.

The experiments with cyanogen bromide suggested that there were six methionine residues per molecule of β-lactamase. Reaction of β-lactamase with tetranitromethane caused a 32% nitration of tyrosine and a single nitrotyrosine-containing peptide was isolated. This suggests that the β-lactamase molecule contains three tyrosine residues. If the methionine and tyrosine contents of β-lactamase correspond to 6 and 3 residues, respectively, then a molecular weight of 2.0×10^4 daltons is indicated for β-lactamase. The tryptophan content of the protein was estimated as three residues per molecule, assuming this molecular weight. The extinction coefficient of the protein at 280 nm correlated with a total of three residues of tyrosine and three of tryptophan per molecule.

A molecular weight of 2.0×10^4 daltons corresponds to a sequence containing about 180 amino-acids. The summation of the sequences represented by tryptic peptides exceeds this figure slightly but there is some duplication of sequences amongst the well-characterised tryptic peptides and this phenomenon probably occurs in other tryptic peptides.

The possibility remains that only 65% of a 230-residue protein was represented in the tryptic peptides. It was calculated that 70% to 80% of the products of tryptic digestion were subjected to the initial fractionation and so an insoluble region of the protein left after tryptic digestion and accounting for 35% of the sequence is unlikely. The weight of evidence lies in favour of a molecular weight of about 2.0×10^4 daltons, but further confirmatory experiments are desirable.

Dale and Smith (1971b) have obtained values of $2.0 - 2.1 \times 10^4$ daltons for the molecular weight of the β-lactamase from E.coli K12

containing the TEM RTF, using gel filtration and ultracentrifugation methods.

Purified E.coli β-lactamase was analysed for amino-acids. The calculation of the number of residues per molecule from the analytical data has been discussed above and in Chapter II. The limitations of the analytical technique have been discussed in Chapter IV. An accurate amino-acid composition waits upon the completion of the amino-acid sequence, which will also enable an accurate molecular weight to be calculated. It has been calculated that E.coli β-lactamase contains one residue of cysteine per molecule.

Another factor contributing to the inaccuracy of the amino-acid composition was the free amino-acid content of the β-lactamase preparation. Free serine was purified from tryptic, chymotryptic and cyanogen bromide hydrolysates of the protein. Glycine and alanine were also sometimes found. Steven and Tristram (1962) discovered that free serine and, to a lesser extent, other amino-acids and small peptides were associated with human skin collagen after a lengthy purification procedure. Meadway (1969a) observed a discrepancy in the glutamic acid content determined by analysis of B.licheniformis β-lactamase, when compared to the amino-acid sequence. The purification of the enzyme from a culture filtrate rich in glutamic acid was suggested to be the source of free glutamic acid bound to the protein. A small peptide isolated during sequence studies on S.aureus β-lactamase did not fit the sequence but its structure suggested that it was derived from the bacterial cell wall mucopeptide (R.P. Ambler, personal communication).

The variability of the carbohydrate content of the E.coli β-lactamase preparation suggested that it too was a non-covalently-

bound contaminant of the preparation. In one tryptic digest, a fraction which was both ninhydrin- and carbohydrate-positive was shown to consist of free reducing monosaccharide and free amino-acids.

Jack and Richmond (1970) have completed an extensive comparative survey of β-lactamases from Gram-negative organisms and have classified them into eight types, on the basis of substrate profile, sensitivity to various inhibitors and electrophoretic mobility. The β-lactamase Ia and Ib purified from a strain of E.coli and the E.coli R1 β-lactamase (Linqvist and Nordstrom, 1970) (Yamugishi et al., 1969; Sawai et al., 1970) are all coded for by resistance transfer factors faund in E.coli. These enzymes, together with that from E.coli W3310, can be classified as Type 1 β-lactamases (Jack and Richmond, 1970). They all have molecular weights in the region of 2.0 - 2.2 x 10^4 daltons.

Dale and Smith (1971b) have studied several Gram-negative β-lactamases and defined two groups. One group contains β-lactamases specified by R-factors in two strains of E.coli (including E.coli K12 containing the TEM RTF), two strains of Klebsiella and in Aerobacter. These enzymes all have molecular weights in the range 1.8 - 2.3 x 10^4 daltons. The other group containing two chromosomally-mediated β-lactamases from E.coli 419 and 214T and these enzymes have molecular weights of 3.1 and 3.2 x 10^4 daltons respectively. These latter enzymes are comparable with the E.coli K12 and E.coli DH131 chromosomally-mediated β-lactamases (Lindstrom et al., 1970; Neu and Winschell, 1970; see Table 1, Chapter I).

Most of the well-characterised, RTF-mediated β-lactamases from Gram-negative bacteria have similar molecular weights in the

region of 1.8 - 2.3 x 10^4 daltons. One exception, the enzyme from E.coli K12 bearing the R-1818 RTF has a molecular weight of about twice this figure (Dale, 1971). These enzymes may form a related group analogous to the Gram-positive β-lactamases.

Most of the well-characterised, chromosomally-mediated β-lactamases from Gram-negative bacteria fall into a distinct group, typified by a molecular weight of around 3.0 x 10^4 daltons.

The N-terminus and the C-terminus of E.coli β-lactamase have been determined and correlated with fragments of the amino-acid sequence. The experiments with fluorodintrobenzene were fairly successful although the yields of Dnp-leucine were rather low. Hofmann (1964) observed a low reactivity of the N-terminal leucine of a cyanogen bromide peptide from trypsinogen with FDNB. He suggested that the formation of ε-Dnp-lysine at the N-terminal region of the peptide would render it extremely hydrophobic and therefore unreactive. The N-terminal sequence of the peptide was:

leu-ile-lys-leu-lys-

It is interesting that leucine amino-peptidase did not release amino-acids from this peptide. A similar phenomenon would not be expected with β-lactamase in view of the postulated N-terminal sequence.

Gray (1967) has emphasised that the reaction of proteins with 1-dimethylaminonaphthalene-5-sulphonyl chloride may often give poor yields of α-Dns-amino-acids and has suggested (Gray, 1972a) the use of SDS to maintain the protein in solution during the reaction. A similar technique (K. Weber, unpublished) was used in the present work, but with limited success.

The potential use of 4-sulphophenylisothiocyanate in the sequential degradation of polypeptides has been demonstrated. Much more development of the technique is necessary. It may be useful for determining the sequence of the first few residues in a protein or large peptide and thus simplify the recognition of peptides from the N-terminus in subsequent proteolytic digests. Neither this technique, nor the automatic sequential degradation procedure (Edman, 1970) yielded any information on the N-terminal sequence of E.coli β-lactamase.

E.coli β-lactamase was hydrolysed with DPCC-trypsin, SBTI-chymotrypsin and pepsin, and also with cyanogen bromide and with DPCC-trypsin after the chemical blocking of lysine residues with ETPA. It was considered that most of the tryptic peptides were isolated and characterised. An extensive characterisation of the peptides produced by some other protease might suffice to complete the amino-acid sequence of E.coli β-lactamase but this was not accomplished in the present work. The evidence for six fragments, which account for about 60% of the molecule, together with some tentative overlaps which extend these fragments, has been presented.

No well-defined examples of unusual peptide bonds being hydrolysed by trypsin were observed. There were several cases of slow cleavage by trypsin at bonds adjacent to polar residues (Hill, 1965). The use of ETPA (Riley and Perham, 1970) to block lysine residues and restrict tryptic cleavage to the C-terminus of arginine residues complicated tryptic digestion, possibly due to incomplete blocking and unblocking reactions.

The results obtained with chymotryptic and peptic peptides do not permit a review of the specificity of the respective proteases.

There was no evidence for cleavage of β-lactamase other than C-terminal to methionine residues by cyanogen bromide. The X7 CNBr peptides (Chapter VIII) confirmed sequences within the tryptic peptides T2d and T14e and peptide X6 (fragment 6) was a 17-residue peptide incorporating the C-terminus of the whole protein. The remaining peptides were only partly characterised. The relative insolubility of these peptides rendered them difficult to purify and to sub-digest. In retrospect, the use of SDS-acrylamide gel electrophoresis to check the purity of the large CNBr peptides would have been advisable. The use of maleic anhydride or ETPA as a disaggregating agent (Bruton and Hartley, 1968) might have improved the fractionation of these peptides by gel filtration.

The use of cation-exchange chromatography as the first stage in the purification of proteolytic digests of β-lactamase was more successful than the use of gel filtration, but extensive purification of peptides by paper electrophoresis and chromatography was necessary in any case. The yields of peptides were low and variable. In several cases the complexity of digests was aggravated by the poor cutting of fractions from gel filtration experiments, leading to the occurrence of the same peptide in several fractions. Deamidation (e.g. T6a/T9a) and isomerisation of α-aspartic acid (e.g. T2d/T4a) contributed to the complexity of at least some of the proteolytic digests. No significant ninhydrin-negative peptides were detected, except in the ETP-β-lactamase experiment. Possibly these peptides, if present, were masked by ninhydrin-positive peptides in the early purification stages and hence were lost.

There was some evidence for the retention of peptides con-

taining aromatic residues during gel filtration (Determann and Walter, 1968). The gel filtration of the peptic peptides on Sephadex G-25 in 0.1 M-ammonia was also interesting as the acidic peptides were preferentially excluded from the gel (see Table 17, Chapter VII). This phenomenon was observed by Gelotte (1960).

The amino-acid analyses were reported in mols of amino-acid per mol of peptide, to one decimal place, with impurities greater than 0.1 mol/mol also reported. Some analyses were marred by analyser failure or gross impurity. Where possible, these results have been reported and interpreted, with suitable qualifications. Several examples of low yields of particular amino-acids have already been discussed.

As regards the techniques of sequence determination, one drawback of this work has been the commencing of analytical experiments on peptides subsequently shown to be impure. The choice between absolute purity and sufficient recovery for subsequent experiments was an ever-present dilemma which was especially acute in dealing with peptides produced by sub-digestion.

The use of thin layer chromatography on polyamide layers to identify Dns-amino-acids (Woods and Wang, 1967) is more sensitive than the electrophoretic technique used in the present work, and might have permitted more extensive sequential degradation.

The time and material used in the experiments with CNBr were not justified by the results obtained. In retrospect, they could have been better employed in "labelling" the cysteine residue with ^{14}C-iodoacetic acid and the tryptophan residues with 2-hydroxy-5-nitrobenzyl bromide (Horton and Koshland, 1965), and isolating the corresponding tryptic peptides. The isolation of the N-terminal

tryptic peptide after treatment of the protein with FDNB (Ambler, 1963a) would have been a satisfactory confirmation of the N-terminal sequence. In view of the frequent short overlaps between tryptic peptides and other peptides produced by proteolysis, a thermolytic digest of the protein (Ambler and Meadway, 1968) might have yielded useful peptides.

The six fragments were compared with the S.aureus and B. licheniformis sequences by both visual and automatic techniques. Fragment 1 appears to be homologous with residues 129-157 in the S.aureus sequence and with residues 135-163 in the B.licheniformis sequence, about 35% of the residues being identical. No other homologies were indicated but it was estimated that identity of 15% or less would not have been detected.

E.coli β-lactamase contains a single tyrosine residue which is especially reactive with tetranitromethane. Nitration of this tyrosine residue results in a 36% loss of β-lactamase activity. This is comparable with the specific nitration of tyrosine-77 in B.licheniformis β-lactamase which results in a 40% loss of activity (Meadway, 1969a), with the specific nitration of tyrosine-71 in S.aureus (R.P. Ambler, unpublished) and with the specific iodination of a single tyrosine residue in B.cereus β-lactamase which results in a 30-35% loss in activity (Csanyi et al., 1971). The partial sequence of the nitrotyrosine-containing peptide suggests that the sequence around the reactive tyrosine residue may be similar to but not identical with the corresponding sequences of the S.aureus, B.licheniformis and B.cereus sequences.

E.coli β-lactamase contains a histidine residue which is especially reactive with iodoacetic acid and which is essential for

β-lactamase activity. The tryptic peptide containing this residue is remarkably similar to a tryptic peptide in the B.licheniformis β-lactamase, six residues being identical and the remaining three being chemically similar. B.cereus β-lactamase is somewhat less similar in this region and S.aureus β-lactamase has a tyrosine residue (tyrosine-79) in place of the histidine residue.

Large sections of the E.coli β-lactamase sequence show less than 15% identity with the corresponding proteins from Gram-positive organisms. A few regions of the E.coli β-lactamase sequence are much more similar with parts of the completed sequences but it is not known if these regions are in corresponding positions relative to the rest of the primary structure.

An analogous situation, which has been more fully resolved, is presented by the so-called serine proteases. A range of enzymes of mammalian and microbial origin all serve to hydrolyse peptide bonds in proteins, though with differing specificities. The use of some of these enzymes in the present work partly illustrates this specificity range. The enzymes all have a common feature in that all are sensitive to inhibition by DFP, which phosphorylates a serine residue essential to the catalytic activity in each case. An essential histidine residue is another common feature (Dayhoff et al., 1969).

Sequence homology between trypsinogen, chymotrypsinogens A and B and parts of elastase were reported by Hartley et al (1965), who noted that small clusters of residues scattered throughout the sequences were identical. These observations were extended to include chymotrypsinogen C, the full sequence of elastase, the partial sequence of the thrombin B chain and some species variants

(Hartley and Shotton, 1971). The various chymotrypsinogens were closely related. Trypsinogen, chymotrypsinogen and elastase exhibited about 40% homology with each other, and parts of the thrombin sequence were about 30% identical with the other sequences. Only 11 residues, in a total of about 260, were invariant in all of the sequences. The tertiary structures and mechanisms of action of some of these enzymes have been shown to be very similar (Blow et al., 1969; Keil,1971; Hartley and Shotton, 1971).

The sequences containing the reactive serine and histidine residues, and the disulphide bridges of a protease from Streptomyces griseus have been determined. 50% identity between sequences representing about one-quarter of the molecule and the corresponding sequences in trypsinogen has been demonstrated (Jurasek et al., 1969). A slight similarity between the sequence of the α-lytic protease from Myxobacter 495 (Sorangium; Olsen et al., 1970) and the trypsin family suggests that all of the serine proteases so far discussed may have arisen by divergent evolution from a common ancestral enzyme. The proof of this hypothesis will depend upon the discovery of other homologous proteins in other species and the construction of a phylogenetic tree. The possibility remains that the sequence similarities between the mammalian and bacterial serine proteases are a result of convergent evolution.

Another group of serine proteases of bacterial origin, the subtilisins, contain reactive serine and histidine residues but are not otherwise similar to the mammalian serine proteases. It is of interest that two subtilisins derived from different strains of Bacillus subtilis only exhibit 70% sequence similarity (Smith et al., 1966). Even in this case, there may be discovered serine

proteases the sequences of which are intermediate between the homologous group and the subtilisins.

The first lesson to be drawn from this analogy is that even when the complete sequence of the E.coli β-lactamase is available, its evolutionary relationship with the β-lactamases from Gram-positive organisms may not be clear. The similarities detected in the present study are probably greater than between the subtilisins and the mammalian proteases or between the Myxobacter protease and the mammalian proteases. It may be tentatively concluded that the E.coli β-lactamase and the β-lactamases from Gram-positive bacteria have evolved from a common ancestor. The homology between the E.coli sequence and the others will almost certainly be less than between the S.aureus and B.licheniformis sequences but in at least one region the B.licheniformis (and the B.cereus) sequence may resemble the E.coli sequence more than the S.aureus sequence.

The division of bacteria into Gram-positive and Gram-negative is perhaps the most fundamental classification of this phylum. It is based upon a difference in cell wall and cell surface structure which implies that the probability of the physical transfer of genetic material occurring between Gram-positive and Gram-negative bacteria is low, even if the transferred material could be expressed, propagated and integrated into the host chromosome. No examples of this phenomenon have been reported to date (Jones and Sneath, 1970). Thus β-lactamase, or a protein from which β-lactamase has evolved, may have been present in a primitive ancestor of modern bacteria, before the evolution of the differences which are detected by the Gram stain. However the existence of regions of sequence in which

Gram-positive and Gram-negative bacteria are more similar that two different genera of Gram-positive bacteria may imply that genetic transfer across this barrier has occurred in the past. If this possibility, even if very rare, is admitted, then the corresponding possibilities of the transfer and hybridisation of β-lactamase genes between many species of bacteria must be considered. The evolution of the β-lactamase sequence could then, perhaps, be divorced from bacterial phylogeny and considered in terms of relationships between enzyme structure and function.

The evidence for the involvement of a single tyrosine and a single histidine residue in the activity of E.coli β-lactamase is fairly conclusive, but the effects of the modification of other residues were not examined. The cysteine residue may be of particular interest. There must certainly be other residues involved in the binding of penicillin to the enzyme. Pollock (1965) showed that B.licheniformis 6346C produced a β-lactamase which had a lower molecular activity and K_m than the β-lactamase produced by the standard strain, B.licheniformis 749C. The amino-acid sequence of the 6346C variant differs from the other at only two positions; a glutamine residue replaces arginine-163 and a valine residue replaces methionine-259 (R.P. Ambler, unpublished). One or both of these residues is involved in the activity of the B.licheniformis enzyme, over and above the involvement of tyrosine-77 and the presumed involvement of histidine-84. It is interesting that V/K_m (the "physiological efficiency", Pollock, 1965) is the same for the 6346C and 749C variants. In view of the nature of the $-(\log V/pK_m)$ vs pH graph found in the present work, this may mean that the substitutions in the 6346C enzyme do not affect the

binding between the ensyme and the substrate. Pollock (1965) has remarked on the similarity of the physiological efficiencies of several β-lactamases; this may reflect a similar mode of binding between the enzyme and the substrate.

One of the main drawbacks of the extension of these results to a mechanism of action of the enzyme is the fact that the reactive residues have not been shown to be in juxtaposition. A satisfactory demonstration of this phenomenon by chemical methods will be very difficult, although preliminary experiments, not reported in the text, have shown that lysine and tyrosine residues in E.coli β-lactamase will react with 1,5-difluoro-2,4-dinitrobenzene under conditions which might be expected to form intramolecular bridges (Cuatrecasas et al., 1968), and with a partial loss of activity. These studies were not pursued but might give valuable information as to the three-dimensional interrelationships of a completed one-dimensional structure. The determination of the three-dimensional structure by X-ray crystallography is the most reliable method of confirming the position of the active site. Nevertheless, the partial loss of activity in acid and the elimination of this effect in the partially-active nitro-β-lactamase suggested that the kinetic data might be correlated with the results of chemical modification.

The postulated mechanism is the simplest one which fits the available evidence. There is no evidence in model systems for an imidazole group acting as a general acid catalyst (Gutfreund and Knowles, 1967) but imidazole, having a pK near 7, is well suited to this type of reaction at neutral pH values, since both protonation and deprotonation are relatively rapid (Eigen and Hammes, 1963).

It might be possible to test the mechanism by observing the effect of deuterium oxide on the reaction; if proton transfer is rate-limiting, then the reaction will be much slower in D_2O but Gutfreund (1970) and Knowles/have emphasised that D_2O may alter the pH and the pKs of ionising groups.

The proposed mechanism is unlike the CDM model for penicillin hydrolysis (Kinget and Schwartz, 1969) and more closely resembles the model for concerted acid-base catalysis of the mutarotation of α-tetra-O-methyl-D-glucose by 2-hydroxypyridine (Swan and Brown, 1952), in which the hydroxyl and pyridinium groups were thought to be oriented so as to permit simultaneous acid and base catalysis.

The large-scale production of E.coli β-lactamase is now straightforward and this should aid the completion of the amino-acid sequence and further chemical and enzymological studies. Although preliminary experiments have failed to produce crystals of the enzyme (L. Sawyer, personal communication) it remains a good candidate for the determination of tertiary structure by X-ray crystallography. Such a project involving the S.aureus β-lactamase is now in motion. In view of the enzymological results of the present work, a similar study in S.aureus might prove very fruitful in the near future, since the tertiary structure would be the ultimate test of any mechanistic theories.

The study of the sequences of other RTF-mediated β-lactamases from Gram-negative organisms may now be feasible. The relationships between a range of proteins specified by a transferable genetic element should be very interesting. These enzymes, reviewed earlier in this chapter, may be very similar and homology with the E.coli β-lactamase may considerably aid sequence determination.

Particular examples, such as the Aerobacter cloacae 53 β-lactamase, which is extremely sensitive to p-chloromercuribenzoate (Jack, 1971) would be especially interesting with respect to the relationship between structure and function.

With a known evolutionary relationship between the β-lactamases from Gram-positive bacteria and the E.coli W3310 β-lactamases, the phylogeny of other Gram-negative β-lactamases could perhaps be estimated on the basis of immunological cross-reaction between them and the E.coli enzyme. Prager and Wilson (1971a,b) have claimed that the "immunological distance" between two proteins is approximately proportional to the difference in their amino-acid sequence, up to a maximum difference of 30-40%. Cocks and Wilson (1972) have used this technique to construct a phylogenetic tree for enterobacterial alkaline phosphatase. This tree parallels the phylogeny of the bacteria, as deduced by several other methods.

Recent observations show that resistance to penicillins has been acquired by the pathogenic Pseudomonas aeruginosa in several clinical isolates. Subsequently it was shown that this resistance was due to the acquisition of RTFs which specified the synthesis of β-lactamase (Lowbury et al., 1969; Sykes and Richmond, 1970). Should this spread of resistance continue, then it is conceivable that β-lactamase evolution may be monitored and that a deeper knowledge of the structure and function of β-lactamase may be of great practical importance.

REFERENCES

Acher, R., Fromageot, C. and Jutisz, M. (1950). Biochim. Biophys. Acta 5, 81-88.

Ambler, R.P. (1963a). Biochem. J. 89, 341-349.

Ambler, R.P. (1963b). Biochem. J. 89, 349-378.

Ambler, R.P. (1965). Biochem. J. 96,

Ambler, R.P. (1971). In Recent Developments in the Chemical Study of Protein Structures, 289-305. Institut National de la Sonte et de la Recherche Medicale, Paris.

Ambler, R.P. (1972). Methods Enzymol. XXV, 262-272.

Ambler, R.P. and Brown, L.H. (1967). Biochem. J. 104, 784-825.

Ambler, R.P. and Meadway, R.J. (1968). Biochem. J. 109, 893-895.

Ambler, R.P. and Meadway, R.J. (1969). Nature (London) 222, 24-26.

Andrews, P. (1964). Biochem. J. 91, 222-233.

Anfinsen, C.B., Cuatrecasas, P. and Taniuchi, H. (1971). in The Enzymes IV, 177-204, ed. Boyer, P.D. Academic Press, New York, 3rd ed.

Armstrong, M.D. (1949). J. Amer. Chem. Soc. 71, 3399-3402

Beavan, G.H. and Holiday, E.R. (1952). Advances in Protein Chemistry 7, 319-386.

Bennet, J.C. (1967). Methods Enzymol. XI, 211-213.

Benson, J.V. and Patterson, J.A. (1965). Anal. Chem. 37, 1108-1110.

Bergmann, M. and Fruton, J.S. (1941). Advan. Enzymol. 1, 63-98.

Birr, C., Reitel, C. and Wieland, T. (1970). Angew. Chim. 82, 771.

Blombäck, B. (1967). Methods Enzymol. XI, 398-411.

Blow, D.M., Birktoft, J.J. and Hartley, B.S. (1969). Nature (London) 221, 337-340.

Bornstein, P. and Piez, K.A. (1966). Biochemistry 5, 3460-3473.

Boulton, A.A. and Bush, I.E. (1964). Biochem. J. 92, 11-12.

Braunitzer, G., Schrank, B. and Ruhfus, A., with Peterson, S. and Peterson, U. (1971). Hoppe-Seyler's Z. Physiol. Chem. 352, 1730-1732.

Brown, J.R. and Hartley, B.S. (1966). Biochem. J. 101, 214-228.

Bruice, T.C. and Schmir, G.L. (1957). J. Amer. Chem. Soc. 79, 1663-1667.

Bruton, C.J. and Hartley, B.S. (1968). Biochem. J. 108, 281-288.

Butler, P.J.G. and Hartley, B.S. (1972). Methods Enzymol. XXV, 191-199.

Chauvet, J.P.aand Acher, R. (1972). Biochemistry 11, 916-927.

Citri, N. (1972). in The Enzymes IV, 23-46, ed. P.D. Boyer. Academic Press, New York.

Citri, N. and Zyk, N. (1965). Biochim. Biophys. Acta 99, 427-441.

Cleland, W.W. (1970). in The Enzymes 11, 1-65, ed. Boyer, P.D. Academic Press, New York, 3rd ed.

Click, E.M. and Bornstein, P. (1970). Biochemistry 9, 4699-4706.

Cocks, G.T. and Wilson, A.C. (1972). J. Bacteriol. 110, 793-802.

Cohen, J.A., Oosterbaan, R.A. and Berends, F. (1967). Methods Enzymol. XI, 686-702.

Crestfield, A.M., Moore, S. and Stein, W.H. (1963). J. Biol. Chem. 238, 622-627.

Crestfield, A.M., Stein, W.H. and Moore, S. (1963). J. Biol. Chem. 238, 2413-2420.

Cuatrecasas, P., Fuchs, S. and Anfinsen, C.B. (1968). J. Biol. Chem. 244, 406-412.

Dale, J.W. (1971). Biochem. J. 123, 501-506.

Dale, J.W. and Smith, J.T. (1971a). Biochem. J. 123, 493-500.

Dale, J.W. and Smith, J.T. (1971b). Biochem. J. 123, 507-512.

Dalgleish, C.E. (1952). Biochem. J. 52, 3-14.

Datta, N. and Kontomichalou, P. (1965). Nature (London) 208, 239-241.

Datta, N. and Richmond, M.H. (1966). Biochem. J. 98, 204-209.

Dawson, R.M.C., Elliot, D.C., Elliot, W.H, and Jones, K.M. (1959). Data for Biochemical Research, 205-211. Oxford.

Dayhoff, M.O. and Eck, R.V. (1971). The Atlas of Protein Sequence and Structure. National Biomedical Research Foundation, Silver Spring, Maryland, U.S.A.

Dayhoff, M.O., Hunt, L.T., Hardman, J.K. and Barker, W.C. (1969). in The Atlas of Protein Sequence and Structure, 47-59.

Dent, C.E. (1947). Biochem. J. 41, 240-253.

Determann, H. and Walter, I. (1968). Nature (London) 219, 604-605.

Devor, A.W. (1950). J. Amer. Chem. Soc. 72, 2008-2012.

Dixon, M. (1955). Biochem. J. 55, 161-169.

Dixon, M. and Webb, E.C. (1964). in Enzymes, 116-145. Longmans, London, 2nd ed.

Edman, P. (1970). in Protein Sequence Determination, 211-255 ed. Needleman, S.B.. Chapman and Hall, London.

Eigen, M. and Hammes, G.G. (1963). Advan. Enzymol. 25, 1-38.

Erlanger, B.F. and Cohen, W. (1969). J. Amer. Chem. Soc. 85, 348-349.

Fischer, E. (1894). Ber. dtsch. chem. Ges. 27, 2985-2995.

Fraenkel-Conrat, H., Harris, J.I. and Levy, A.L. (1955). Methods in Biochemical Analysis 22, 259-425.

Frank, G. and Braunitzer, G. (1967). Hoppe-Seyler's Z. Physiol. Chem. 348, 1691-1692.

Gelotte, B. (1960). J. Chromatogr. 3, 330-342.

Goldner, M., Glass, D.G. and Fleming, P.C. (1968). Can. J. Microbiol. 14, 139-145.

Gomori, G. (1955). Methods Enzymol. 1, 143.

Gray, W.R. (1967). Methods Enzymol. XI, 139-151.

Gray, W.R. (1972a). Methods Enzymol. XXV, 121-138.

Gray, W.R. (1972b). Methods Enzymol. XXV, 333-344.

Greenaway, P.J. (1970). Ph.D. Thesis, University of Edinburgh.

Gross, D. (1960). J. Chromatogr. 5, 194-206.

Gross, E. and Witkop, B. (1962). J. Biol. Chem. 237, 1856-1860.

Gundlach, H.G., Stein, W.H. and Moore, S. (1959). J. Biol. Chem. 234, 1754-1760.

Gurd, F.R.N. (1967). Methods Enzymol. XI, 532-541.

Gutfreund, H. and Knowles, J.R. (1967). in Essays in Biochemistry 3, 25-72. ed. Campbell, P,N. and Greville, G.D. Academic Press, New York.

Hamilton, P.B. (1963). Anal. Chem. 35, 2055-2064.

Hamilton, T.H. (1967). Process and Pattern in Evolution. Macmillan, New York.

Harfenist, E.J. (1953). J. Amer. Chem. Soc. 75, 5528-5533.

Harris, J.I., Sanger, F. and Naughton, M.A. (1956). Arch. Biochem. Biophys. 65, 427-438.

Hartley, B.S., Brown, J.R., Kaufman, D.L. and Smillie, L.B. (1965). Nature (London) 207, 1157-1159.

Hartley, B.S. and Shotton, D.M. (1971). in The Enzymes III, 323-373, ed. Boyer, P.D. Academic Press, New York.

Hartsuck, J.A. and Lipscomb, W.N. (1971). in The Enzymes III, 1-56, ed. Boyer, P.D. Academic Press, New York.

Hedges, R.W. (1972). Heredity 28, 39-48.

Heinrikson, R.L., Stein, W.H., Crestfield, A.M. and Moore, S. (1965). J. Biol. Chem. 240, 2921-2934.

Helinksi, D.R. and Yanofsky, C. (1962). Biochem. Biophys. Acta 63, 10-19.

Hill, P. (1972). J. Gen. Microbiol. 70, 243-252.

Hill, R.L. (1965). Advances in Protein Chemistry 20, 37-107.

Hill, R.L. and Smith, E.L. (1957). J. Biol. Chem. 228, 577-599.

Hirs, C.H.W. (1956). J. Biol. Chem. 219, 611-621.

Hoare, D.G. (1972). Nature (London) 236, 437-440.

Hofmann, T. (1964). Biochemistry 3, 356-364.

Horton, H.R. and Koshland, D.E. (1965). J. Amer. Chem. Soc. 87, 1126-1132.

Ikenaka, T., Hammerlin, H., Kaufmann, H. and Schmid, K. (1966). J. Biol. Chem. 241, 5560-5563.

Jack, G.W. (1971). Biochem. Biophys. Acta 250, 428-436.

Jack, G.W. and Richmond, M.H. (1970). J. Gen. Microbiol. 61, 43-61.

Jansen, E.F. and Balls, A.K. (1952). J. Biol. Chem. 194, 721-727.

Jepson, J.B. and Smith, J. (1955). Nature (London) 172, 1100-1101.

Jones, D. and Sneath, P.H.A. (1970). Bacteriological Reviews 34, 40-81.

Jukes, T.H. (1966). Molecules and Evolution. Columbia University Press, New York.

Jurasek, L., Fackre, D. and Smillie, L.B. (1969). Biochem. Biophys. Res. Commun. 37, 99-105.

Keil, B. (1971). in The Enzymes III, 250-275, ed. Boyer, P.D. Academic Press, New York.

Keil-Dlouhá, V., Zylbir, N., Imhoff, J-M., Tong, N-T and Keil, B. (1971). FEBS Lett. 16, 291-295.

Kenkare, V.W. and Richards, F.M. (1966). J. Biol. Chem. 241, 3197-3206.

Kinget, R.D. and Schwartz, M.A. (1969). J. Pharm. Sci. 58, 1102-1105.

Kogut, M., Pollock, M.R. and Tridgell, E.J. (1956). Biochem. J. 62, 391-401.

Koshland, D.E. (1959). in The Enzymes I, 305-346, ed. Boyer, P.D., Lardy, H. and Myrbäck, K. Academic Press, New York, 2nd ed.

Koshland, D.E., Strumeyer, D,H. and Ray, W.J. (1962). Brookhaven Symposia in Biology 15, 101-133.

Kuwabara, S. and Abraham, E.P. (1970). Biochem. J. 115, 859-861.

Léonis, J., Li, C.H. and Chung, D. (1959). J. Amer. Chem. Soc. 81, 419-423.

Levy, A.L. (1954). Nature (London) 174, 126-127.

Levy, A.L. and Chung, D. (1954). Anal. Chem. 25, 396-399.

Linderstrøm-Lang, K. (1952). Lane Medical Lectures, 1951 (II) in K. Linderstrøm-Lang: Selected Papers (1962) 406-426. Danish Science Press, Copenhagen.

Lindqvist, R.C. and Nordstrom, K. (1970). J. Bacteriol. 101, 232-239.

Lindström, E.B., Boman, H.G. and Steele, B.B. (1970). J. Bacteriol. 101, 218-231.

Lineweaver, H. and Burk, D. (1934). J. Amer. Chem. Soc. 56, 658-666.

Lowbury, E.L.J., Kidson, A., Lilly, H.A., Ayliffe, G.A.J. and Jones, R.J. (1969). The Lancet ii, 448-452.

Lowry, O.H., Roseborough, N.J., Farr, A.L., and Randall, R.J. (1951). J. Biol. Chem. 193, 265-275.

Maroux, S., Rovery, M. and Desnuelle, P. (1962). Biochim. Biophys. Acta 56, 202-204.

Meadway, R.J. (1969a). Ph.D. Thesis, University of Edinburgh.

Meadway, R.J. (1969b). Biochem. J. 115, 12P.

Meynell, E., Meynell, G.G. and Datta, N. (1969). Bacteriological Reviews 32, 55-83.

Michl, H. (1951). Monatsch. Chem. 82, 489-493.

Milstein, C., Clegg, J.B. and Jarvis, J.M. (1968). Biochem. J. 110, 631-654.

Milstein, C.P. and Milstein, C. (1968). Biochem. J. 109, 93-99.

Milstein, C. and Sanger, F. (1961). Biochem. J. 79, 456-469.

Morris, H.R., Williams, D.H. and Ambler, R.P. (1971). Biochem. J. 125, 189-201.

Naughton, M.A. and Hagiopan, H. (1962). Anal. Biochem. 3, 276-284.

Naughton, M.A., Sanger, F., Hartley, B.S. and Shaw, D.C. (1960). Biochem. J. 77, 149-163.

Neu, H.C. and Winschell, E.B. (1970). Arch. Biochem. Biophys. 139, 278-298.

Noltman, E.A., Mahowold, T.S. and Kuby, S.A. (1962). J. Biol. Chem. 237, 1146-1154.

Novick, R.P. (1962). Biochem. J. 83, 236-240.

Offord, R.E. (1966). Nature (London) 211, 591-593.

Olsen, J., Nagabhushan, N., Dzwiniel, M., Smillie, L.B. and Whitaker, D.R. (1970). Nature (London) 228, 438-442.

Park, J. (1966). Symp. Soc. Gen. Microbiol. 16, 70-81.

Perret, C.J. (1954). Nature (London) 174, 1012-1013.

Perutz, M.F. and Lehmann, H. (1968). Nature (London) 219, 902-909.

Pollock, H.R. (1957). Biochem. J. 66, 419-428.

Pollock, M.R. (1965). Biochem. J. 94, 666-675.

Pollock, M.R. (1967). Brit. Med. J. 4, 71-77.

Pollock, M.R. (1968). Ann. N.Y. Acad. Sci. 151, 502-515.

Pollock, M.R. (1971). Proc. Roy. Soc. Ser. B. 179, 385-401.

Pollock, M.R. and Torriani, A-M. (1953). C.R. Acad. Sci. 237, 276-278.

Potts, J.T. (1967). Methods Enzym ol. XI, 648-664.

Prager, E.M. and Wilson, A.C. (1971a). J. Biol. Chem. 246, 5979-5989.

Prager, E.M. and Wilson, A.C. (1971b). J. Biol. Chem. 246, 7010-7017.

Ray, W.J. and Koshland, B.E. (1960). Brookhaven Symposia in Biology 13, 135-148.

Richmond, M.H. (1963). Biochem. J. 88, 452-459.

Richmond, M.H. (1965). Biochem. J. 94, 584-593.

Riley, M. and Perham, R.N. (1970). Biochem. J. 118, 733-739.

Riordan, J.F. and Vallee, B.L. (1972). Methods Enzymol. XXV, 515-521.

Rydon, H.N. and Smith, P.W.G. (1952). Nature (London) 169, 922.

Ryle, A.P., Sanger, F., Smith, L.F. and Kitai, R. (1955). Biochem. J. 60, 541-556.

Sanger, F. (1945). Biochem. J. 39, 507-515.

Sanger, F, (1952). Advances in Protein Chemistry VII, 1-67.

Sanger, F. and E.O.P. Thompson (1963). Biochim. Biophys. Acta 71, 468-471.

Sawai, T., Takahashi, K., Yamagishi, S. and Mitsuhashi, S. (1970). J. Bacteriol. 104, 620-629.

Saz, A.K. (1970). J. Cell. Physiol. 76, 397-404]

Schmer, G. and Kreil, G. (1969). Anal. Biochem. 29, 186-192.

Schroeder, W.A. (1967). Methods Enzymol. XI, 351-361.

Schroeder, W.A., Shelton, J.R., Shelton, J.B., Cormick, J. and Jones, R.T. (1963). Biochemistry 2, 992-1008.

Schwartz, M.A. (1965). J. Pharm. Sci. 54, 1308-1312.

Schwartz, M.A. and Pflug, G.R. (1967). J. Pharm. Sci. 56, 1459-1464.

Sherratt, D.J. (1969). Ph.D. Thesis, University of Edinburgh.

Smith, E.L., Markland, F.S., Kaspar, C.B., Delange, R.J., Landon, M. and Evans, W.H. (1966). J. Biol. Chem. 241, 5974-5976.

Smith, J.M.B. and Marples, M.J. (1964). Nature (London) 201, 844.

Smith, J.T. (1963). Nature (London) 197, 900.

Smith, J.T. and Hamilton-Miller, J.M.T. (1963). Nature (London) 197, 769-770.

Smithies, O. (1955). Biochem. J. 61, 629-641.

Smyth, D.G., Stein, W.H. and Moore, S. (1963). J. Biol. Chem. 238, 227-234.

Snell, D.T. and Offord, R.E. (1972). Biochem. J. 127, 167-178.

Sokolovsky, M., Harell, D. and Riordan, J.F. (1969). Biochemistry 8, 4740-4745.

Sokolovsky, M., Riordan, J.F. and Vallee, B.L. (1966). Biochemistry 5, 3582-3589.

Spackman, D.H., Stein, W.H. and Moore, S. (1958). Anal. Chem. 30, 1190-1206.

Spies, J.R. and Chambers, D.C. (1948). Anal. Chem. 20, 30-39.

Steven, F.F. and Tristram, G.R. (1962). Biochem. J. 83, 240-244.

Strominger, J.L., Blumberg, P.M., Suginaka, H., Umbreit, J. and Wickus, G.G. (1971). Proc. Roy. Soc. Ser. B. 179, 369-383.

Swain, C.G. and Brown, J.F. (1952). J. Amer. Chem. Soc. 74, 2538-2543.

Swallow, D.L. and Abraham, E.P. (1958). Biochem. J. 70, 364-373.

Sykes, R.B. and Richmond, M.H. (1970). Nature (London) 226, 952-954.

Toennies, G. and Kolb, J.J. (1951). Anal. Chem. 23, 823-826.

Tung, J.S. and Knight, C.A. (1971). Biochem. Biophys. Res. Commun. 42, 1117-1121.

Van Orden, H.O. and Carpenter, F.M. (1964). Biochem. Biophys. Res. Commun. 14, 399-403.

Waley, S.G. and Watson, J. (1953). Biochem. J. 55, 328-337.

Walsh, K.A. and Brown, J.R. (1962). Biochim. Biophys. Acta 58, 596-598.

Weber, K. and Osborn, M. (1969). J. Biol. Chem. 244, 4406-4412.

Weil, L., Gordon, W.G. and Buchert, A.R. (1951). Arch. Biochem. Biophys. 33, 90-109.

Weil, L., James, S. and Buchert, A.R. (1953). Arch. Biochem. Biophys. 46, 266-278.

Wilson, H. and Cannan, R.K. (1937). J. Biol. Chem. 119, 309-331.

Woods, K.R. and Wang, K-T. (1967). Biochim. Biophys. Acta 133, 369-370.

Yamagishi, S., O'Hara, K., Sawai, T. and Mitsuhashi, S. (1969).
J. Biochem. (Tokyo) 66, 11-20.

Yang, C.A. (1967). in Protein Sequence Determination p.89,
ed. Needleman, S.B. Chapman and Hall, London.

Preparation of Gram-Quantities of a Purified R-Factor-Mediated Penicillinase from Escherichia coli Strain W3310

By J. MELLING

Microbiological Research Establishment, Porton, Salisbury, Wilts., U.K.

and G.J. SCOTT

Department of Molecular Biology, University of Edinburgh, Kings Buildings, Mayfield Road, Edinburgh EH9 3JR, U.K.

(Received 11 May 1972)

Synopsis

Purified penicillinase in gram-quantities, has been prepared from Escherichia coli strain W3310 by using methods developed to handle large amounts of material. The final product had a specific enzyme activity of 3.08 units/μg protein which was over twice as high as that reported previously (Datta & Richmond, 1966). The purified enzyme was similar to that from E. coli strain TEM, but different in molecular weight and some other respects. The difference observed may be a result of the greater purity obtained.

Production and purification processes for R-factor-mediated penicillinases from Escherichia coli have been described by Datta & Richmond (1966), Sawai et al. (1970) and Lindquist & Nordstrom (1970). The enzyme yields/ml of cultur were 200 and 600 in the case of Datta & Richmond (1966) and Sawai et al. (1970) respectively. The units used by Lindquist & Nordstrom (1970) are not comparabl However, the quantities of purified enzymes produced were small; 1.8 mg from 3 litres of culture (Datta & Richmond, 1966), 15.7 mg from 10 litres of culture (Sawai et al., 1970) and 0.64 mg from 12 litres of culture (Lindquist & Nordstrom, 1970).

In the experiments reported in this paper units of penicillinase activity are expressed as μmol of substrate transformed/min at 30°C and pH 7.0. However it should be noted that other authors with whose work comparisons are made (Datta & Richmond, 1966; Sawai et al., 1970; Melling & Ford, 1971) used the unit defined by Pollock & Torriani (1953) which relates activity to μmol of substate transformed/h at 30° and pH 7.0. Thus 60 of the units defined by Pollock & Torriani (1953) are equivalent to one of the present international enzyme units.

To allow a detailed appraisal of the characteristics of a penicillinase mediated by the TEM R-factor (Datta & Kontomichalou, 1965), including the amino acid sequence, much larger quantities of the enzyme were required than had been produced hitherto.

Yields of 5000 enzyme units/ml of culture were obtained (Melling & Ford, 1971) from E. coli strain W3310, which carried the TEM R-factor (M.H. Richmond, personal communication). This corresponds to 1000 units/mg dry wt. of bacteria and thus the preparation of gram amounts of purified enzyme required kilogram quantities of bacteria as a starting material. The procedures reported here were therefore developed for work on a large scale.

Experimental

Culture methods.

E. coli strain W3310 containing the TEM R-factor was obtained from

Professor M.H. Richmond, Dept of Bacteriology, University of Bristol. The organism was maintained on 1% nutrient agar plates (Novick, 1962) containing 25 of ampicillin/ml. Cultures for penicillinase production were grown in the medi described by Novick (1962) at 37°C in a 400-litre fermenter. The fermenter (76 cm diam. x 137 cm high) was fitted with four baffles (6.3 cm x 72 cm) space vertically at equal distances around the vessel wall. The stirrer was fitted with two 30.5 cm impellors, each having four blades 4.4 cm high x 8.3 cm long, extending to the periphery of the impellor disc. The general construction of t culture vessel and associated equipment was similar to the 100-litre vessel described by Elsworth & Stockwell (1968). The pH was automatically maintained at 6.8 using phosphoric acid and the cultures were aerated with 300 litres of air/min and stirred at 250 revs/min. When the penicillinase titre reached a maximum, after about 15 h of incubation, the bacteria were collected by centri-fugation and washed with 0.1M sodium phosphate buffer pH 7.0. The washed cell paste was stored at -20°C until required.

General methods

Enzyme assays. Penicillinase assays using sodium benzyl penicillin as substrate were by the micro-iodometric method of Novick (1962) or by a spectro-photometric variation of the Perret (1954) assay (Sherratt, 1969). Units of penicillinase activity are expressed as µmol of substrate transformed/min at 30 and pH 7.0.

Protein estimation. Protein was normally estimated by the method of Lowry et al., 1951, with bovine serum albumin as the standard. Elution of protein from columns was followed by measuring the E254.

Ion exchange celluloses. DEAE-cellulose, DE 11 and DE 52 obtained from Whatman Products, Reeve Angell, London EC4, UK were prepared for use according to the makers directions.

Sephadex. Sephadex G-75 purchased from Pharmacia, Uppsala, Sweden was use according to the makers directions.

Starch-gel electrophoresis. This was done by the method of Smithies (1955

using Connaught hydrolysed starch obtained from Connaught Medical Research laboratories, Toronto, Ontario, Canada.

Molecular weights. Determinations of molecular weights were performed by gel filtration using Sephadex G-75 (superfine grade) (Andrews, 1964) and by electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate (Weber & Osborn, 1969).

Amino acid analysis. The method of Spackman et al. (1958) with a temperature of 105°C for hydrolysis was employed using a Model 120c Amino acid analyse (Beckman Instruments Inc, Palo Alto, Calif USA). Tryptophan was determined by the method of Spies & Chambers (1948). Cysteine was determined by reacting the protein with iodoacetic acid (BDH Ltd, Poole, Dorset, UK) and estimating carboxymethyl cysteine in an acid hydrolysate of the protein (Gurd, 1967).

Carbohydrate. The carbohydrate content of protein samples was determined by the method of Devor (1950).

Results

Release of the penicillinase

The penicillinase in E. coli strain W3310 is cell-associated, with little in the culture supernatant; therefore a cell-breaking procedure was required. Datta & Richmond (1966) used ultrasonic treatment, but this was not suitable in the present case where multi-kilogram quantities of bacteria were involved. A Manton-Gaulin homogenizer (APV Ltd, Crawley, Sussex, UK) operated at 50 litres/ was used for bacterial disruption. Maximum release of the penicillinase occurred after one pass of an aqueous suspension of bacteria (10% dry weight) through the machine at a pressure of 55.12 MN/m^2 (8000 psi), as shown in Table

Purification of the penicillinase

The purification was done in two parts; first, the processing of the broken cell suspension to give about 5×10^6 units of penicillinase with a specific enzyme activity of 283 units/mg of protein, then purification in batch of 1.6×10^6 enzyme units to give material with a specific enzyme activity of 3083 units/mg of protein. The two parts of the process are summarized in Table 2 & 3.

Part I

Centrifuguation. The broken cell suspension had a high viscosity and was treated with 0.4 µg deoxyribonuclease/ml before centrifugation to improve the recovery of supernatant (Melling & Atkinson, 1972). The suspension was centrifuged at 13000 g at 8°C and a flow rate of 20 litres/h in a Sharples no 6 open clarifier.

Ammonium sulphate fractionation. The supernatant was fractionated with ammonium sulphate and Fig. 1 shows the results of a small-scale process. In the routine preparation solid ammonium sulphate (May & Baker reagent grade) was added to the supernatant to give a 20% (w/v) solution. This was centrifuged as above, the deposit discarded and the ammonium sulphate content of the supernatant increased to 56% (w/v). The resulting deposit, collected by centrifugation, contained the penicillinase which was stable in this condition for at least six months when stored at 4°C.

Dialysis. The ammonium sulphate precipitate was dissolved in water and dialysed against demineralized water in a continuous dialyser (Heppell Engineering, Harlow, Essex, UK) to decrease the conductivity to 1000 µsiemens. The enzyme solution was then dialysed to equilibration with the appropriate buffer system. For small-scale work dialysis tubing was used.

Absorption and elution from DE 11 cellulose. For ease and speed of operation it was intended to use a batch absorption and elution process.

Initial observations indicated that the conditions used by Datta & Richmon (1966) ie. 0.01M sodium phosphate buffer at pH 7.0, gave relatively poor absorption of the enzyme. Only 1.83×10^3 units of penicillinase were absorbed g of dry DE 11. Therefore to have absorbed the normal batch of about 6.6×10^6 units would have required 4-5 kg of DE 11 and involved very large elution volumes.

The results in Table 4 show that increasing the pH of the phosphate buffer to 8.2 improved absorption; a reduction in buffer concentration further increased the amount of enzyme absorbed. However, the use of tris buffer (Table 4)

produced a marked improvement in absorption capacity. For routine use, 0.02 M-tris buffer pH 8.2 was the system selected since although the absorption capacit was higher at pH 9.0 the enzyme could not be eluted by increasing the buffer concentration at this pH.

In the large-scale purification process 500g of DE 11 was equilibrated to 0.02 M-tris buffer, pH 8.2 and added to the enzyme solution. The suspension was stirred for 30 min and the DE 11 collected by filtration and washed three times with 5 litre volumes of 0.02 M-tris buffer, pH 8.2. This removed unabsorbed protein and pigment. The cellulose was then treated with successive 1.5 litre volumes of 0.3 M-tris buffer, pH 8.2 and the eluates collected by filtration after 30 min of stirring.

Dialysis and freeze drying. The eluates from the DE 11 were pooled (see Table 2), dialysed continuously against 0.02 M-tris buffer, pH 8.2 and freeze-dried in 7.5 litre batches. The freeze-dried material was stored at -20°C until required

Part II

Dialysis. A typical batch containing about 1.6×10^6 units of enzyme activity was dissolved in water and dialysed against 6 x 8 litre volumes of water at 4°C; when the pH of this solution rose to about pH 8.5 it was adjusted to pH 7 with 1 M-HCl. Ultimately the solution was adjusted to pH 7.0 with 1 M-HCl and to a conductivity of 80 μsiemens with 0.05 M-tris-HCl pH 7.0.

Chromatography on DE 52 cellulose. The solution of penicillinase was applied at 150 ml/h to a column (20 cm x 2.5 cm) of DEAE-cellulose previously equilibrated to 0.02 M-tris-HCl buffer at pH 7.0; no enzyme was detected in the eluate at this stage. The column was washed with 250 ml of 0.02 M-tris-HCl pH 7.0 and a linear gradient consisting of 350 ml of 0.02 M buffer and 350 ml of 0.05 M buff was applied. Fractions (10 ml) were collected. The pooled fractions from this column were dialysed exhaustively against distilled water at 4°C and freeze-dried; no detectable loss of enzyme activity occurred on freeze-drying.

Separation through Sephadex G-75. The freeze-dried material was dissolved in 10.0 ml of 0.04 M sodium phosphate buffer pH 7.0 and applied to a column (90 cm

x 6 cm) of G-75 Sephadex which had previously been equilibrated and packed in 0.04 M sodium phosphate buffer pH 7.0. This buffer was pumped through the column at 100 ml/h and 8.0 ml fractions were collected. The elution profile is shown in Fig. 2. The pooled fractions were dialysed and adjusted to pH 7.0 and a conductivity of 220 μsiemens with 0.1 M-sodium phosphate buffer pH 7.0.

Rechromatography on DE 52-cellulose. The above solution was applied to a column (10 cm x 2.5 cm) of DEAE-cellulose equilibrated in 0.002 M sodium phosphate buffer pH 7.0. The column was washed with 8mM buffer and then with a linear gradient consisting of 250 ml of 8mM buffer and 250 ml of 16mM buffer; these molarities were found to be critical for good results. The flow rate was 50 ml/h and 6.0 ml fractions were collected. The elution profile is shown in Fig. 3. The pooled fractions were dialysed against water and freeze-dried. Fig. 4 shows the results of starch-gel electrophoresis of samples of this preparation before and after this final stage.

Properties of the penicillinase

Homogeneity. The purified protein was homogeneous as judged by starch-gel electrophoresis at pH 8.5 and pH 4.0 and by its elution profile in gel filtration and ion-exchange chromatography. A very faint band of contaminating protein of low molecular weight was detected by sodium dodecyl sulphate-polyacrylamide-gel electrophoresis. The total carbohydrate content corresponde to 0.8 mol of reducing sugar/mol of protein.

Enzymic properties. The following characteristics were determined for the purified protein using sodium benzylpenicillin as substrate: specific activity 3083 units/mg of protein; K_m; 12 μM; turnover number, 6×10^4 mol/min per mol of enzyme. The relative activities for benzylpenicillin, ampicillin and ceporin were 100%, 112% and 125% respectively (J. Fleming, personal communication).

Molecular weight. Determinations of molecular weight by gel filtration, sodium dodecyl sulphate-polyacrylamide-gel electrophoresis and calculation from the

amino acid composition gave values of 2.1×10^4, 2.7×10^4 and 2.2×10^4 respectively. The total number of tryptic peptides is in approximate accorda with the calculated value. There are 9 lysine residues and 11 arginine resid per molecule, from which one would expect 21 tryptic peptides. Between 20 an 25 tryptic peptides have been isolated in the course of amino acid sequence analysis. (G.K. Scott, unpublished work).

Amino acid composition. Table 5 gives the amino acid composition of the purif protein. The cysteine content was 0.9 mol of carboxymethyl cysteine/mol of iodoacetic acid-treated protein; the tryptophan content was 3 mol/mol of protein.

Molar extinction coefficient. The purified protein had a molar extinction coefficient of 2.14×10^4. The calculated value on the basis of 3 mol tryptophan and 3 mol tyrosine/mol of protein was 2.08×10^4 (Beaven & Holiday, 1952).

Discussion

The final recovery of purified enzyme from the procedure described above exceeded 100% when compared with the penicillinase content of the broken-cell suspension (Tables 1 and 2). However, in some preliminary experiments dilutio of the homogenized cell suspension before ammonium sulphate fractionation was omitted and there was no increase in enzyme titre such as occurred in the larg scale preparation. These results are consistent with other findings (J. Melli & D. Westmacott, unpublished work) which indicate that this enzyme aggregates, probably with other proteins, and there may thus be shielding of the active site of some enzyme molecules in concentrated protein solutions.

In a small-scale preparation the binding capacity of the DEAE-cellulose m be of minor importance, but in the present case an improvement obtained by replacing phosphate buffer with tris buffer (Table 4) greatly facilitated the large scale process by a ten-fold decrease in the amounts of DEAE-cellulose, a hence of eluate, required. The lower binding-capacity in the presence of phosphate may have resulted from competition for sites between the enzyme and phosphate ions.

The final product was purified to a specific activity over twice as high as that achieved previously (Datta & Richmond, 1966). In pure form, the enzym has a somewhat different Michaelis constant when compared with crude extracts of *E. coli* TEM, but the relative activities of the two preparations with respe to various substrates are almost identical.

Datta & Richmond (1966) concluded that the relatively small inhibitory effect of *p*-chloromercuribenzoate on penicillinase from *E. coli* TEM meant that there were probably no reactive thiol groups in the enzyme. The present study indicates that a cysteine residue is present.

The molecular weight data appear at first sight to be inconclusive, especially when the earlier value of 16700, obtained by ultracentrifugation, i considered (Datta & Richmond, 1966). There is evidence that some proteins giv anomalous results in the gel-filtration method for molecular weight determinat (Andrews, 1964) and some doubt has recently been cast upon mobility in sodium dodecyl sulphate gel-electrophoresis as a criterion of molecular weight (Tung Knight, 1971). The value of 16700 was obtained with a preparation which may w have contained less than 50% penicillinase, and thus may be considerably in error.

If the analytical data and the gel filtration estimate of molecular weigh are considered as independent determinations which give approximately the same result, then a molecular weight of between 21 and 22 x 10^3 is arrived at. In addition the ultrafiltration characteristics of the enzyme also provide eviden for a molecular weight in excess of 20 x 10^3 (J. Melling & D. Westmacott, unpublished work).

The penicillinases Ia and Ib purified by Sawai *et al.* (1970) and Yamagish *et al.* (1969) and the penicillinase from *E. coli* R1 purified by Lindquist & Nordstrom (1970) are all from strains of *E. coli* and are all coded for by gene carried on resistance transfer factors. These enzymes, together with that fro *E. coli* TEM could be classified as Type 1 penicillinases in the system of classification devised by Jack & Richmond (1970). These enzymes all have

molecular weights in the region of 20-22 x 10^3 as estimated by gel filtration. The revised estimates of molecular weight for the penicillinase at present und consideration are in accord with the other penicillinases mentioned above.

References

Andrews, P. (1964). Biochem.J. 91, 222.

Beaven, G.H. & Holiday, E.R. (1952). In Advances in Protein Chemistry, vol VI p. 319. Ed. by Anson, M.L., Bailey, K. & Edsall, J.T. New York: Acadeı Press.

Datta, N. & Kontomichalou, P. (1965). Nature, Lond. 208, 239.

Datta, N. & Richmond, M.H. (1966). Biochem.J. 98, 204.

Devor, J.W. (1950). J.Am.chem.Soc. 72, 2004.

Elsworth, R. & Stockwell, F.T.E. (1968). Process Biochemistry March, 229.

Gurd, F.N. (1967). In Methods in Enzymology, vol XI, 532. Ed. by Colwick, S.P. & Kaplan, N.O. New York: Academic Press Inc.

Jack, G.W. & Richmond, M.H. (1970). J.gen.Microbiol. 61, 43.

Lindquist, R.C. & Nordstrom, K. (1970). J.Bact. 101, 232.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951). J.biol.Chei 193, 265.

Melling, J. & Atkinson, A. (1972). J.appl.Chem.Biotechnol. 22, 739.

Melling, J. & Ford, J.W.S. (1971). J.gen.Microbiol. 65, iv.

Novick, R.P. (1962). Biochem.J. 83, 229.

Perret, C.J. (1954). Nature, Lond. 174, 1012.

Pollock, M.R. & Torriani, A.M. (1953). C.R. Acad.Sci., Paris, 237, 276.

Sawai, T., Takahashi, K., Yamagishi, S. & Mitsuhashi, S. (1970). J.Bact. 104 621.

Sherratt, D.J. (1969). Ph.D. thesis, University of Edinburgh.

Smithies, O. (1955). Biochem.J. 61, 629.

Spackman, D.H., Stein, W.H. & Moore, S. (1958). Anal.Chem. 30, 1190.

Spies, J.R. & Chambers, D.C. (1948). Anal.Chem. 20, 30.

Tung, J.S. & Knight, A. (1971). Biochem.Biophys.Res.Comm. 42, 1117.

Weber, K. & Osborn, M. (1969). J.Biol.Chem. 244, 4406.

Yamagishi, S., O'Hara, K., Sawai, T. & Mitsuhashi, S. (1969). Japan J.Bioche 66, 11.

Table 1. Release of penicillinase from Escherichia coli strain W3310

Samples of an aqueous suspension of E. coli (10% w/v) were passed through a Manton-Gaulin homogenizer operated at various pressures. Penicillinase and protein determinations were made on the supernatant after centrifuging at 10000 g for 15 min.

Operating pressure		Penicillinase (units/ml)		Protein (mg/ml)
MN/m^2	lb/min^2			
	0	2,700	45	2.9
13.78	2000	18,000	300	19.0
27.56	4000	30,600	510	52.3
41.34	6000	35,500	591	63.3
55.12	8000	36,000	600	62.6
68.90	10000	35,100	585	61.3

Experimental details are given in the text.

Stage	Procedure	Amount: Volume (l)	Amount: Weight (g)	10^{-6} × total enzyme activity recovered (units)		Specific enzyme activity (Units/mg of protein)	Recovery (%): per stage		Recovery (%): Overall	
1	Homogenization	12		4.46	~~4.46~~	7.13	100		100	
2	Centrifugation	60		6.41	~~6.41~~	12.25	142		143	
3	1st ammonium sulphate fractionation (20% w/v)	60		9.37	~~9.37~~	51.31	146		209	
4	2nd ammonium sulphate fractionation (56% w/v)		2424	9.23	~~9.23~~	66.83	98		206	
5	Dialysis	8		9.15	~~9.15~~	68.00	99		205	
6	Absorption and elution from DE-11 cellulose									
	(a) 1st 3 pooled eluates	4.5		5.10	6.87	283.33	56	75	114	153
	(b) 2nd 3 pooled eluates	4.5		1.76		91.72	19		39	
7	Freeze drying									
	(a) 1st 3 pooled eluates		145	5.00	6.75	283.33	98	98	111	150
	(b) 2nd 3 pooled eluates		148	1.75		91.67	99		39	

The starting material was a portion of the freeze dried product from stage 7 (Table 1). Experimental details are given in the text.

Stage	Procedure	Amount		10^{-6} × total enzyme activity recovered	10^{-2} × specific enzyme activity (Units/mg of protein)	Recovery (%)	
		Volume (ml)	Weight (mg)			per stage	Overall
8	Dialysis	1100		1.98	1.56	100	100
9	Chromatography on DE-52 cellulose	400		1.87	4.33	94	94
10	Gel filtration on G-75 sephadex	200		1.77	26.50	94	89
11	Chromatography on DE-52 cellulose	80		1.70	30.83	96	85
12	Dialysis and freeze drying		550	1.70	30.83	100	85

Table 4. Effect of pH, concentration and buffer composition on the amount of penicillinase absorbed per gram on DE-11 cellulose

Known amounts of DE-11 cellulose were equilibrated to various buffer systems and then added to solutions of penicillinase which had been dialysed against the appropriate buffer. The penicillinase solutions were assayed before and after 30 min of equilibration with the DE-11 cellulose.

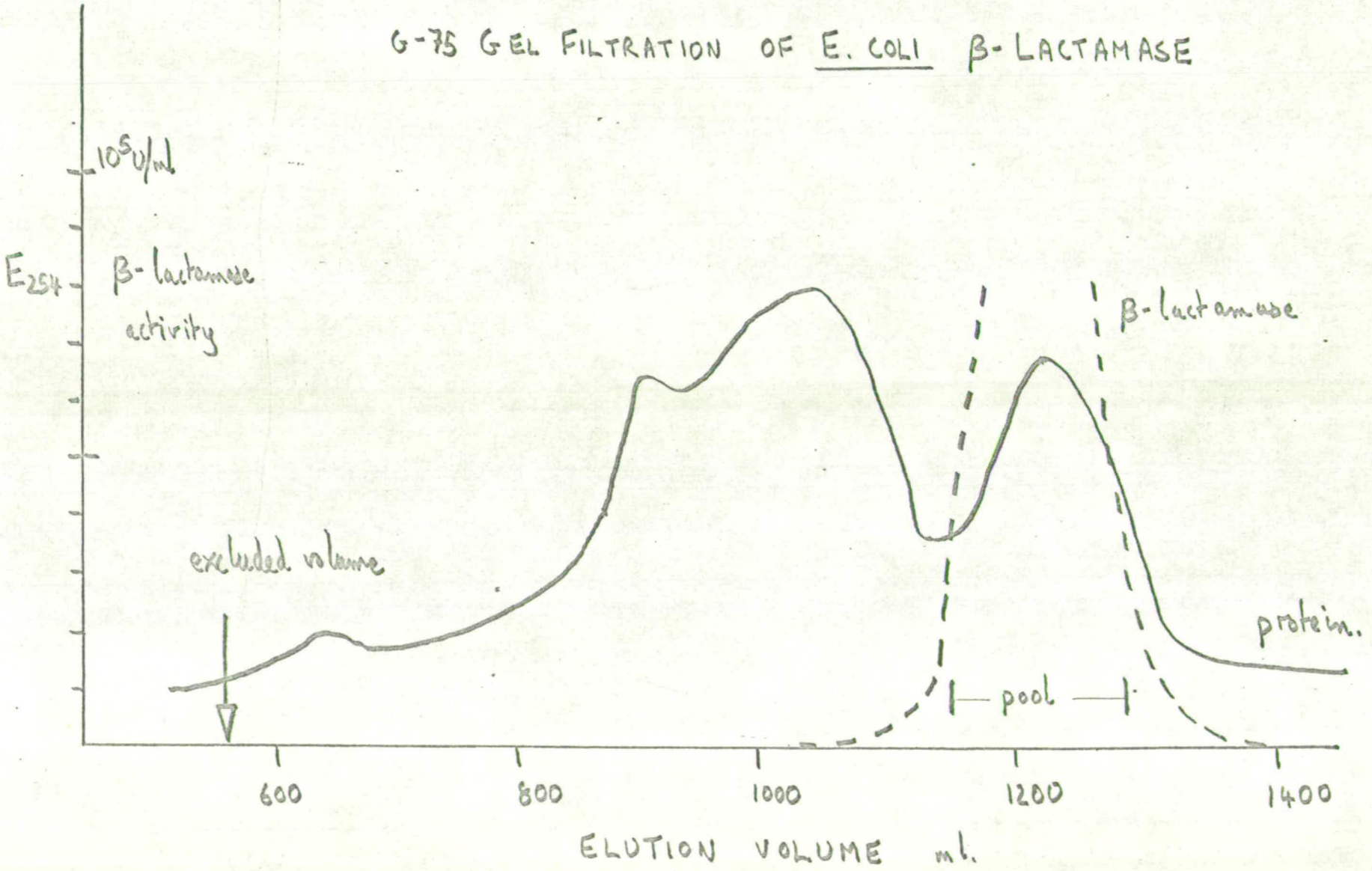
pH	10^3 x Penicillinase units absorbed/g of dry DE-11 cellulose: Sodium phosphate buffer (0.01 M)	Tris buffer (0.02 M)
6.5	1.16	-
7.0	1.83	-
7.5	2.50	-
8.0	3.33	-
8.2	4.17	15.65
9.0	-	26.66

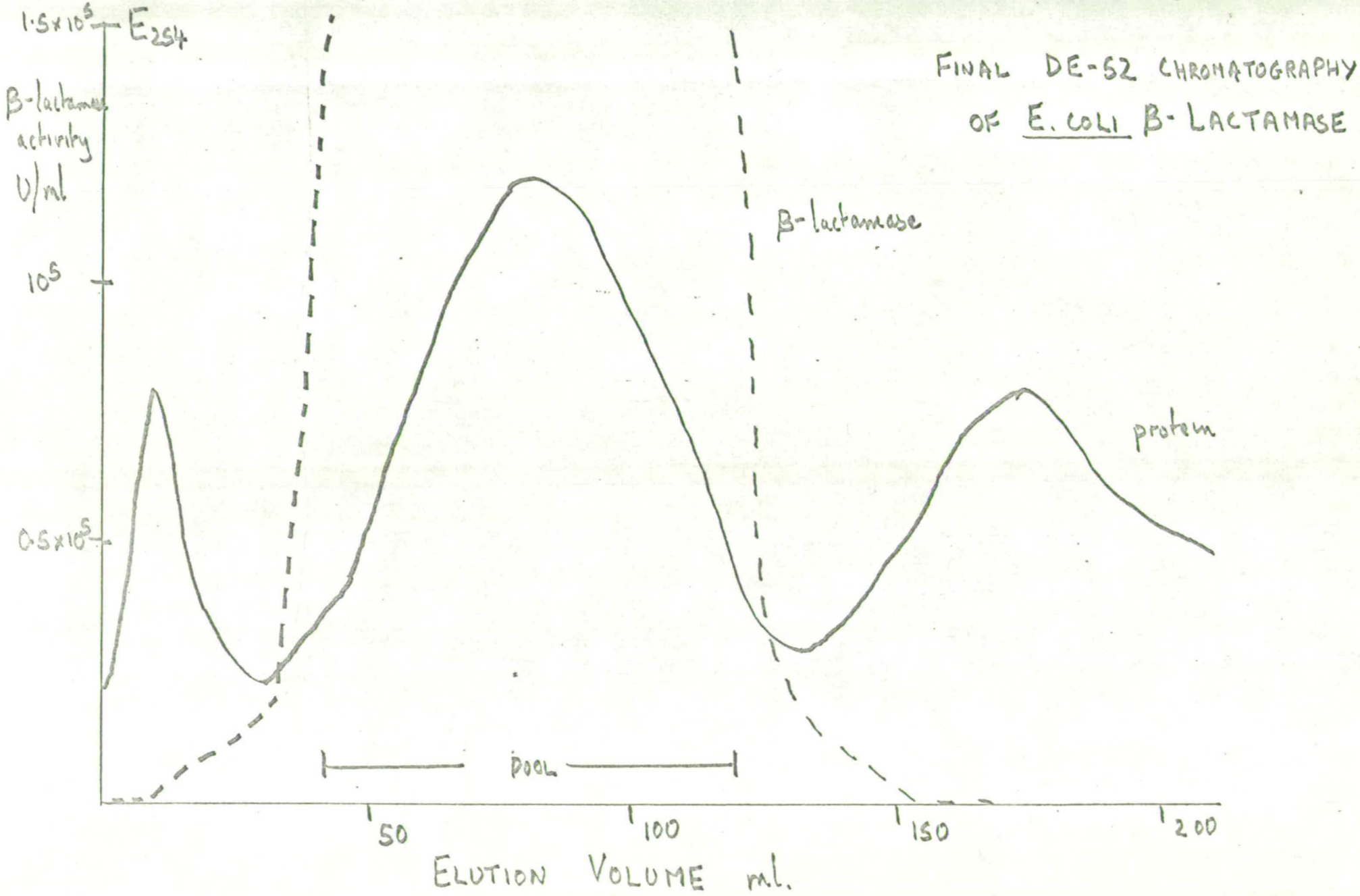
Concentration of phosphate buffer pH 8.2	10^{-3} x Penicillinase units absorbed/g of dry DE-11 cellulose
0.001M	10.33
0.01M	4.17
0.1M	Nil

Table 5. Amino acid composition of penicillinase from Escherichia coli strain W3310

Columns 1 and 2 show the numbers of residues calculated by analysing hydrolysed samples from two different preparations of the enzyme. Results are expressed in number of residues per molecule, assuming a molecular weight of 2.2×10^4. Column 3 gives the mean values to the nearest whole number. The values for threonine and serine were increased by 5% and 10% respectively to correct for losses due to hydrolysis. Methods for analysis are given in the text.

Amino acid	1	2	3
Lys	9.0	9.0	9
His	3.8	4.0	4
Arg	10.4	10.7	11
Asp	17.5	16.8	17
Thr	13.0	13.3	14
Ser	9.6	10.0	11
Glu	19.8	19.5	20
Pro	6.9	7.8	7
Gly	14.6	14.6	15
Ala	17.5	17.2	17
Val	9.2	8.8	9
Met	5.4	5.6	6
He	8.9	8.9	9
Leu	19.8	20.7	20
Tyr	3.1	3.1	3
Phe	4.0	4.0	4
Trp	-	-	3
Cys	-	-	1


G-75 GEL FILTRATION OF E. COLI β-LACTAMASE
10^5 U/ml.
E_{254}
β-lactamase activity
excluded volume
β-lactamase
protein.
pool
600
800
1000
1200
1400
ELUTION VOLUME ml.


FINAL DE-52 CHROMATOGRAPHY
OF E. COLI β-LACTAMASE
1·5×10⁵
E254
β-lactamase activity U/ml
10⁵
0·5×10⁵
β-lactamase
protein
POOL
50
100
150
200
ELUTION VOLUME ml.

APPENDIX II

Extracellular nucleases from *Bacillus licheniformis* 749

The sequences of extracellular nucleases from two strains of *Staphylococcus aureus* have been determined (Taniuchi *et al.*, 1967; Cusumano *et al.*, 1968) and it was felt that the sequence of any corresponding enzyme produced by *Bacillus licheniformis* 749 would considerably extend the comparison between the two organisms afforded by the 40% sequence homology between their respective β-lactamases (Ambler and Meadway, 1969). Accordingly, the identification, purification and characterisation of nuclease activity from culture supernatants of *B.licheniformis* 749 was undertaken.

The DNase assay procedure was based upon that of Anfinsen (Heins *et al.*, 1966b). This procedure was also used, with heat-denatured yeast RNA, for the determination of RNase activity.

The maximum DNase activity found in cultures of *B.licheniformis* was about 1 unit per ml and the resultant yields of pure enzyme were very low. Several unsuccessful attempts were made to generate and isolate mutants which could produce high levels of the enzyme. Mutagenisation was effected with N-nitrosoguanidine (Dubnau and Pollock, 1965) and the plate assays of Gesteland (1966) and Lacks (1970) were employed.

Culture Conditions

Nuclease production by *B.licheniformis* in both rich and defined media was studied. The results are summarised by Figure 1. High levels (ca. 3U/ml) of nuclease could be attained in broth cultures but this declined in the stationary phase of the culture, presumably

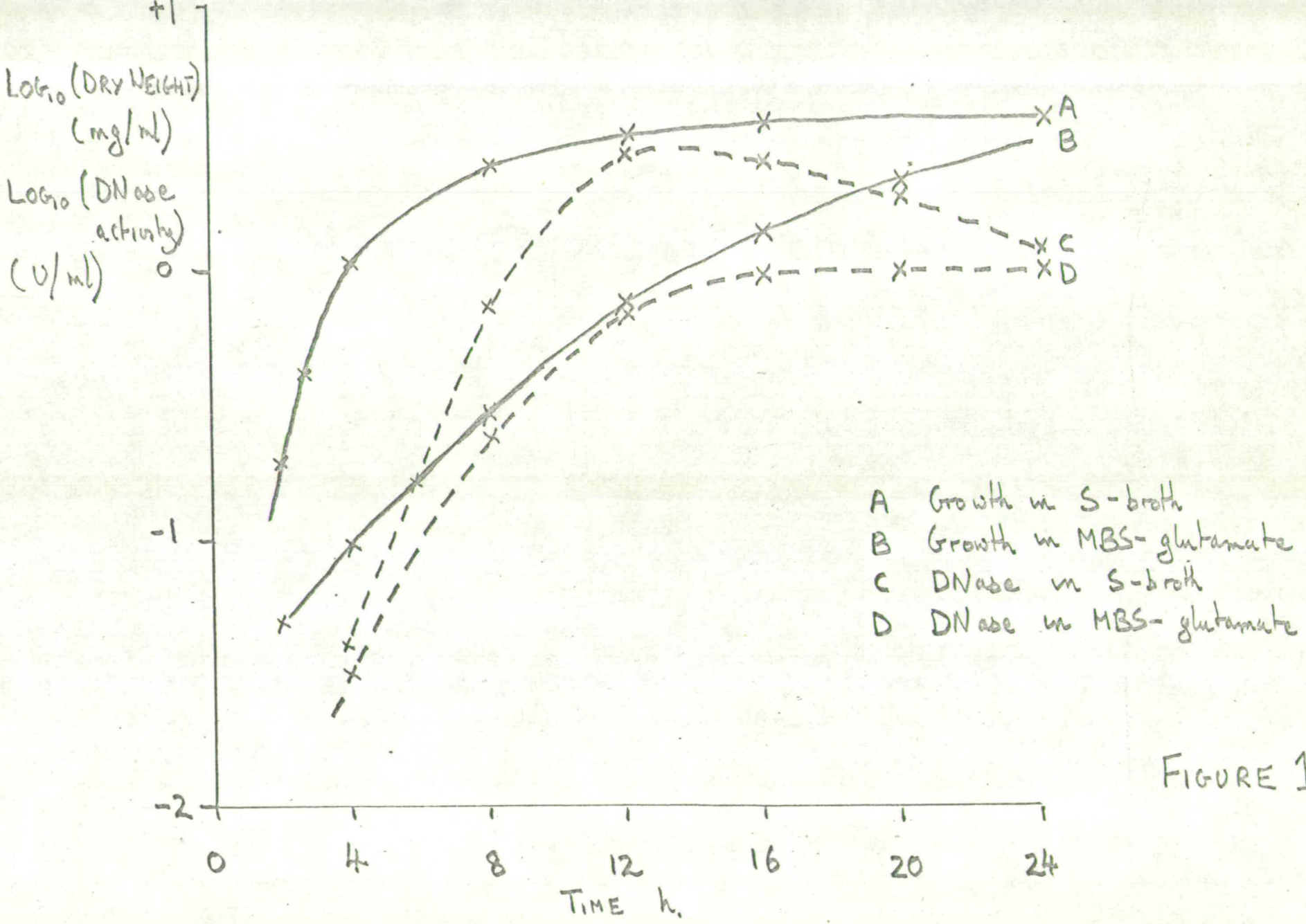

+1
Log10 (Dry Weight) (mg/ml)
Log10 (DNase activity) (U/ml)
0
-1
-2
A
B
C
D
0
4
8
12
16
20
24
Time h.
A Growth in S-broth
B Growth in MBS-glutamate
C DNase in S-broth
D DNase in MBS-glutamate

FIGURE 1.

due to protease activity as sporulation commenced. The higher cost and the greater difficulty in purification of nuclease from broth cultures also influenced the choice of the MBS-glutamate (Meadway, 1969) medium for large-scale cultures. It was possible to produce about 1.0 U/ml of DNase activity at a cell density of about 3 mg/ml (dry weight) in batch cultures of 10 or 50 l. The 10 l cultures were innoculated with a 500 ml culture grown overnight in a shake-flask and normally reached 3 mg/ml in about 24 h. In one case, a culture reached this cell density in about 16 h and the nuclease activity was about 10% of the normal figure.

Birnboim (1966) has observed that protoplasting of B.subtilis causes nuclease to be released into the protoplast supernatant; this enzyme appears to be identical to the extracellular variety. On a small scale, it was found possible to protoplast B.licheniformis using MBS salt 0.75 M in sucrose; this medium is similar to a protoplasting medium used by Chesbro and Lampen (1968). A culture which yielded a total of 220 units of DNase in the culture supernatant was found to release a further 135 units into the protoplast supernatant. No activity was detected in resuspended protoplasts.

Attempts at large-scale protoplasting invariably led to very viscous supernatants, indicative of cell lysis and release of nucleic acids. The danger of contamination by intracellular nucleases precluded any attempt to extract enzymes from these preparations.

DNase activity was found to be sensitive to trypsin; release of the cell-bound fraction with this enzyme, as can be done with cell-bound penicillinase (Pollock, 1965), was not feasible.

TABLE 1.

Initial Purification (DNase activity)

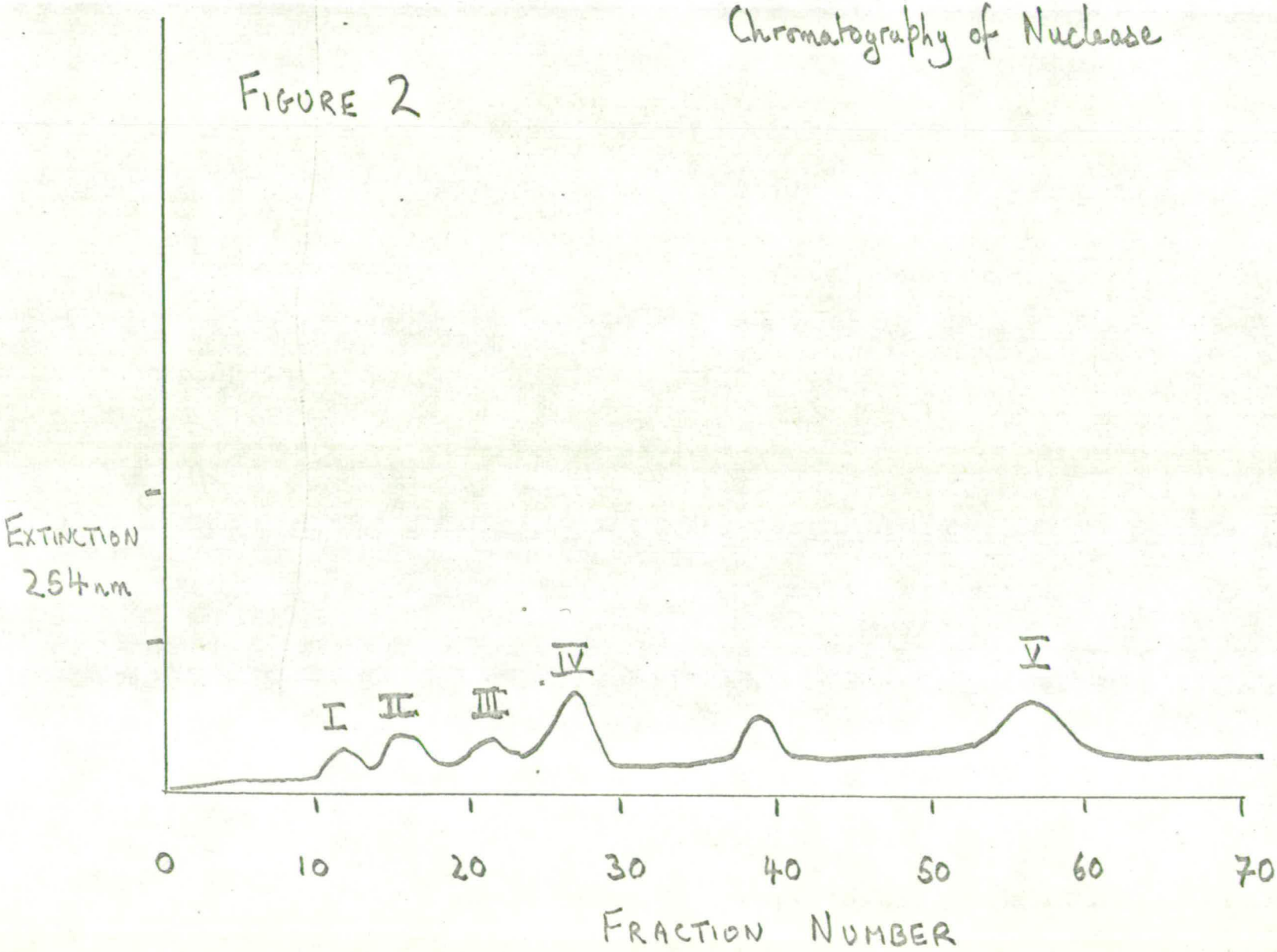
1. 50 litres 45,000 U	Continuous flow centrifugation	Dialysis 48 hr 6,750 U	Elution from DE-11 5,400 U
2. 200 ml 180 U	Centrifugation 12,000 x g 40 min.	Dialysis 24 hr 170 U	-
3. 10 litres 8,700 U	Centrifugation 2,500 x g 2 hr	Dialysis 24 hr 6,100 U	Elution from DE-11 6,400 U
4. 10 litres 9,200 U	Diluted and absorbed to DE-11		Elution from DE-11 6,000 U

TABLE 2.

	DNase		RNase
Culture supernatant	1200 U	total activity	860 U
24 h dialysis 95°C	800 U		
Absorbed to DE-11 Eluted	720 U		
20 h dialysis 15°C	680 U		
DE-52 chromatography			
V. 570 U		Fractions I, II, III, IV	
Rechromatography			
Va. 540 U		Total RNase activity 750 U	
Approx. 0.7 mg of protein.		Fraction IV contained	
Specific activity 780 U/mg.		approx. 1 mg protein and	
About 200 U total RNase activity.		240 U.	
Yield approx. 45%.		Specific activity 240 U/mg.	
Vb. RNase only.			
Not studied further.			

Chromatography of Nuclease

FIGURE 2



Purification

Cultures were passed through an Alfa-Lavall continuous-flow centrifuge to remove the cells. Dialysis of the resulting supernatants against tap-water to lower the conductivity invariably led to considerable losses in activity. High-speed centrifugation prior to dialysis minimised these losses. Dilution of the culture supernatant with tap-water was effective in reducing the conductivity of a 10 l culture but very large volumes were involved.

When the conductivity approximated the 1 mho, the pH was adjusted to pH 8.5 and, the solution was passed through Whatman DE-11 resin, previously equilibrated with 0.01 M-ammomium acetate buffer pH 8.5. Two passages sufficed to absorb more than 95% of the activity. 200 g of DE-11 were sufficient for a 50 l culture supernatant.

The resin was repacked as a convenient column and washed with 0.25 M-ammonium acetate pH 8.5. The nuclease activity could then be eluted with 1.0 M-ammonium acetate or sulphate. Recoveries ranged from 80% to 105%. This data is summarised in Table 1.

It was found that the activity was unstable when freeze-dried. The activity was not fractionated cleanly by ammonium sulphate precipitation. Some of the RNase activity, but none of the DNase, could be absorbed on to CM-cellulose at pH 4.8. This evidence suggested that there were at least two enzymes in the "nuclease" preparation.

However, the residual activity from a lyophilised preparation appeared in a single peak when chromatographed on Sephadex G-100. This was confirmed by chromatography on Sephadex G-50.

The most satisfactory stage in further purification was

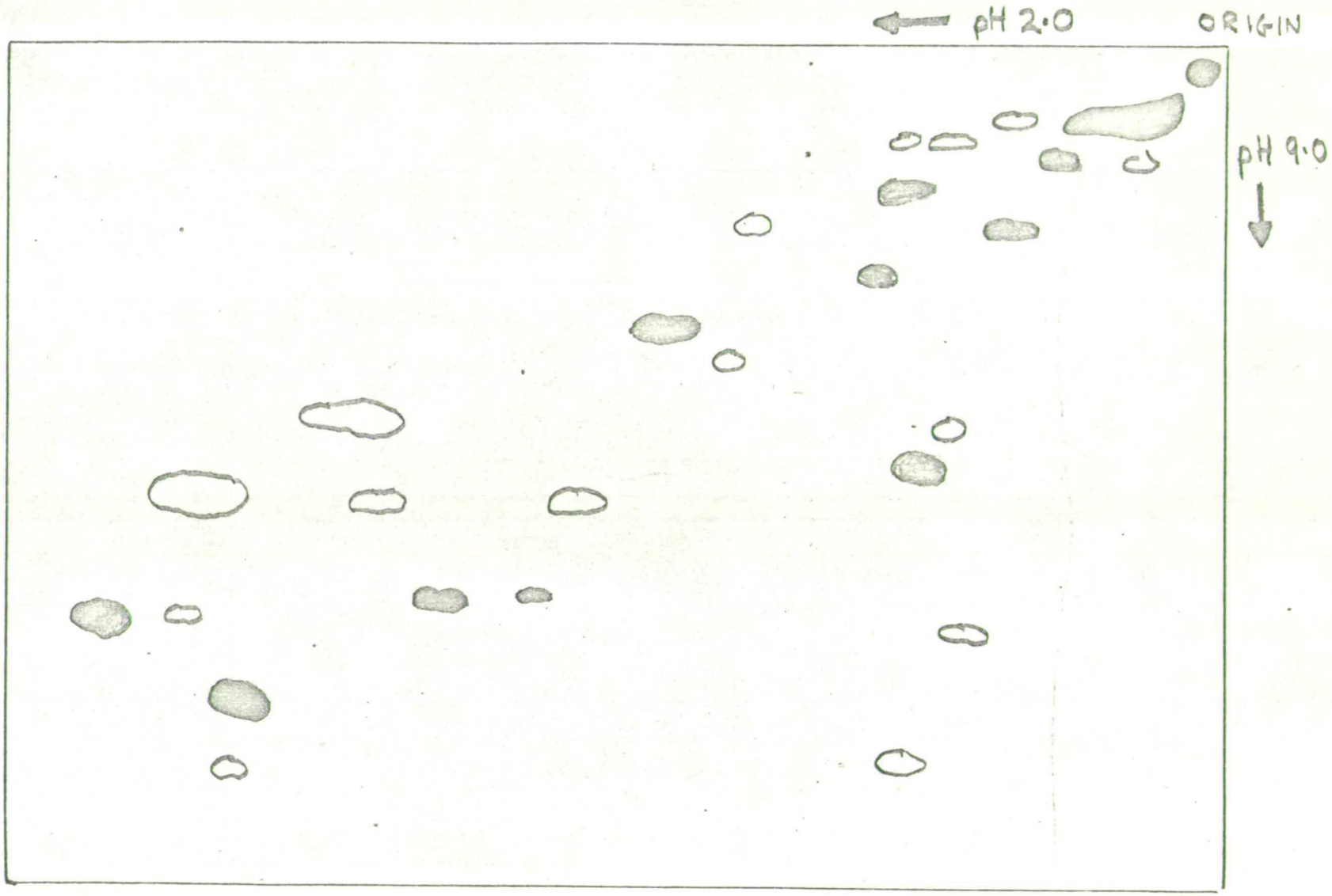


FIGURE 3

dialysis or dilution followed by re-absorption on to a small column of DE-52 in 0.01 M-ammonium acetate pH 8.5. This column was eluted with a linear gradient of ammonium acetate pH 8.5, from 0.01 M to 1.0 M. Five fractions with nuclease activity were produced, as shown in Figure 2. The activity was generally stable when dialysed prior to this stage.

Fractions I, II, III and IV were all RNase enzymes only, with identical specificity towards RNA. Fraction V contained 830 units of DNase and 880 units of RNase activity. Specificity studies indicated exo-DNase and endo-RNase activity. Rechromatography yielded fractions Va and Vb. The former appeared to be an exo-DNase enzyme, with some exo-RNase activity. Fraction Vb was another endo-RNase. This fractionation was successfully reproduced with another preparation. The various fractions appeared to be pure, as judged by starch-gel electrophoresis. The purification is summarised in Table 2.

Properties of Nucleases

The various nuclease fractions could not be separated by Sephadex gel filtration. A sample prior to the final fractionation was passed through a G-50 column and the elution volume was noted. The column was then calibrated with lysozyme, insulin, β-lactoglobulin and blue dextran. The elution volume for nuclease was somewhat smaller than that for insulin. A graph of V_e vs. log (Molecular Weight) gave an approximate molecular weight of 11,000 for nuclease.

The pH optimum was found to be 9.0 and calcium had no effect on the activity or the pH optimum. This was true of both the DNase and the RNase.

An amino-acid analysis was carried out on a sample prior to the final fractionation. In view of the known heterogeneity of this material, the analysis is of little value. Tryptic digestion, with and without performic acid oxidation, followed by one-dimensional electrophoresis showed that oxidation was necessary for complete digestion.

Specificity of Nuclease

Samples of the various nuclease fractions were incubated with ^{32}P-labelled RNA and DNA in capillary tubes. The digests were examined by one-dimensional electrophoresis on amino-ethyl-ion-exchange paper (Whatman) at pH 3.5 and by two-dimensional electrophoresis at pH 9.0 and pH 2.0 on DE-81 paper. Autoradiograms were prepared from the electrophoretograms.

Fractions I to IV proved to have indistinguishable specificities towards RNA, producing various oligonucleotides as well as free adenosine and an adenosyl-uridine dinucleotide. This is shown in Figure 3. There was no digestion of DNA by these fractions.

Fraction V gave very unusual results. The one-dimensional autoradiogram clearly showed that four DNA was hydrolysed to give all four mononucleotides of equal intensities, indicating an exo-nucleolytic cleavage of DNA. The two-dimensional autoradiogram for RNA digestion showed a considerable production of oligonucleotides, indicating an endonucleolytic cleavage of RNA. The one-dimensional RNA autoradiogram showed the four mononucleotides.

The subsequent production of fractions Va and Vb explains these results. Fraction Va is an exonuclease, active against both DNA and RNA. Fraction Vb is an endoribonuclease, although with a different specificity from that of fractions I to IV.

Discussion

The purification of various extracellular nuclease enzymes from B.licheniformis 749 has been achieved. They consist of:-

(i) An exonuclease active against DNA and, to a lesser extent, RNA.

(ii) An endonucleolytic RNase which appears in several chromatographically distinct forms.

(iii) A distinct endonucleolytic RNase. These enzymes appear to have similar molecular weights of around 11,000 and a similar pH optimum.

They are all somewhat labile but can be purified relatively easily. From the data in Table 2, it can be seen that a 50 l culture of B.licheniformis 749 would yield perhaps 30 or 40 mg of pure exonuclease and one or two hundred milligrams of the major endo-RNase.

S.aureus produces a single endonuclease active against both RNA and DNA. It has a pH optimum of about 9.5 and this is lowered to pH 8.5 by calcium ions. It has a molecular weight of 16,800 (Heins et al., 1966a). Thus, there is no functionally-homologous nuclease in B.licheniformis 749 and any chemical homology could well be minimal.

It would be time-consuming and expensive to produce the B.licheniformis nucleases in sufficient quantity for amino-acid sequence determination.

In B.subtilis an "exo-DNase with RNase activity" (Birnboim, 1966) and an endo-RNase (Nishimura, 1966) have been isolated from culture supernatants in similar yields to the B.licheniformis enzymes. The endo-RNase was unstable to prolonged dialysis.

Recent work on the isolation of an autolytic enzyme from *B.subtilis* (Brown *et al.*, 1976) encountered considerable difficulties because of the lability of the enzyme in most of the common purification techniques. The partly purified autolytic enzyme appeared to contain 5% teichoic acid and the problems in purification appeared to be partly due to an undefined association of the protein with cell wall or membrane material. In this context it is noteworthy that the amino-acid analysis of "nuclease" indicated a considerable amino-sugar content.

Birnboim (1966) has shown that most of the *B.subtilis* DNase activity is cell-bound and is released on protoplasting. Thus the *B.subtilis* and *B.licheniformis* nucleases may be cell-bound or "periplasmic" (Neu and Heppel, 1964) and their appearance in the culture supernatant may be as a result of leakage. The variable levels of activity found in culture supernatants are plausible in terms of this theory (Nishimura, 1966; present work). This contrasts with enzymes such as *S.aureus* nuclease and penicillinase which are actively secreted (Omenn and Friedman, 1970; Chesbro and Lampen, 1968).

This work was carried out in 1969/70, before partly-purified *Escherichia coli* β-lactamase was available. I am grateful to Mr. S.G. Hughes, for advice and help with large-scale cultures, and to Dr. K. Murray, who kindly provided advice and materials for the autoradiography experiments.

References

Ambler, R.P. and Meadway, R.J. (1969) Nature, Lond. 222, 24-26.

Birnboim, H.C. (1966) J. Bacteriol. 91, 1004-1011.

Brown, W.C., Frazer, D.K. and Young, F.E. (1970) Biochim. Biophys. Acta 198, 308-315.

Chesbro, W.R. and Lampen, J.O. (1968) J. Bacteriol. 96, 428-437.

Cusumano, C.L., Taniuchi, H. and Anfinsen, C.B. (1968) J. Biol. Chem. 243, 4769-4777.

Dubnau, D.A. and Pollock, M.R. (1965) J. Gen. Microbiol. 41, 7-21.

Gesteland, R.F. (1966) J. Mol. Biol. 16, 67-84.

Heins, J.N., Suriano, J.R., Taniuchi, H. and Anfinsen, C.B. (1966a) J. Biol. Chem. 242, 1016-1020.

Heins, J,N., Taniuchi, H. and Anfinsen, C.B. (1966b) in "Procedures in Nucleic Acid Research" pp.79-84 ed. Cantoni, G.L. and Davies, D.R. Harper and Row, New York, 1966.

Lacks, S. (1970) J. Bacteriol. 101, 373-383.

Meadway, R.J. (1969) Ph.D. Thesis, University of Edinburgh.

Neu, H.C. and Heppel, L.A. (1964) Biochem. Biophys. Res. Comm. 14, 109-112.

Nishimura, S. (1966) in "Procedures in Nucleic Acid Research" pp.56-63 ed. Cantoni, G.L. and Davies, D.R. Harper and Row, New York, 1966.

Omenn, G.S. and Friedman, J. (1970) J. Bacteriol. 101, 921-924.

Pollock, M.R. (1965) Biochem. J. 94, 666-675.

Taniuchi, H., Anfinsen, C.B. and Sodja, A. (1967) J. Biol. Chem. 242, 4736-4751.

APPENDIX III

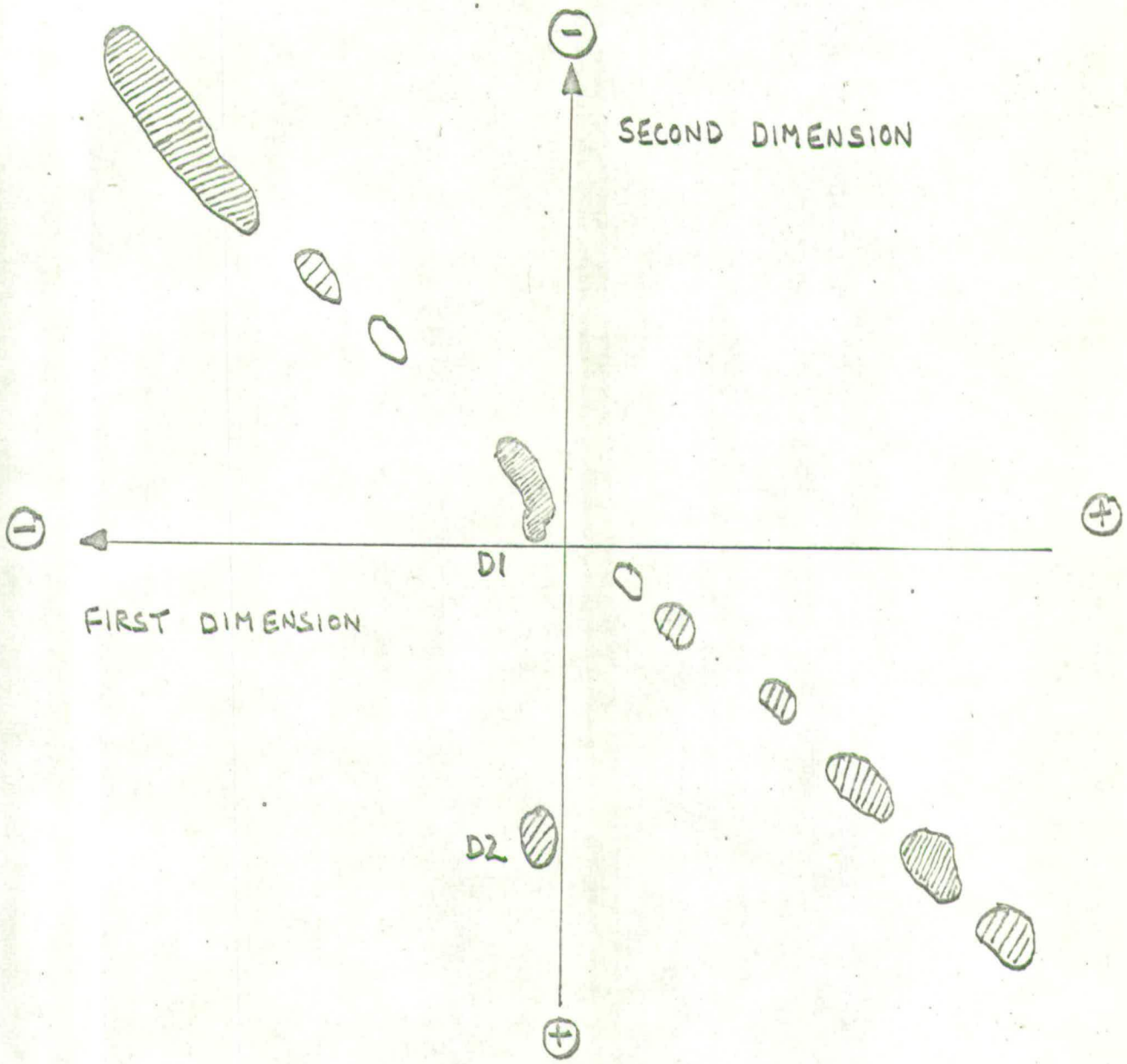
Demonstration of a Single Cysteic Acid-Containing Peptide in β-lactamase

This experiment was based upon the techniques described by Brown and Hartley (1966).

0.5 μmol of native β-lactamase was digested with 0.1 mg of subtilisin for 5 h at pH 8.5 and at 37°C. The freeze-dried products were separated by electrophoresis at pH 6.5 in two 5 cm-wide strips on Whatman 3 mm paper. One strip was incubated for 1 h in an atmosphere of performic acid in a desiccator. When dry, it was cut into two 2.5 cm-wide strips, each of which was sewn into another sheet of 3 mm paper so as to permit pH 6.5 electrophoresis in a direction at right angles to that of the original electrophoresis. One such "diagonal" peptide map was stained with ninhydrin in the normal way and is shown in the diagram. The other was lightly sprayed with 0.05% ninhydrin in acetone and developed at room temperature, in the dark, overnight.

The spots D1 and D2 were eluted from the lightly-stained peptide map but were too weak to permit any structural analysis. The corresponding region in the unoxidised strip was cut out and eluted and seven peptides were isolated after pH 3.5 electrophoresis. Peptide N3 had a mobility of +0.4 at pH 3.5 and a mobility of +0.03 at pH 3.5 after oxidation with performic acid. It was analysed but was too weak for accurate quantitation. Its tentative composition was ($cySO_3$,asx,thr,glx,ala,gly,val,met,ile,leu,lys). No N-terminus was identified.

PERFORMIC ACID DIAGONAL



This experiment indicates that β-lactamase probably contains a single cysteine residue.